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p21-activated kinase1 (Pak1) is a negative regulator of NADPH-oxidase 2 in ventricular myocytes

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Abstract

Ischemic conditions reduce the activity of the p21-activated kinase (Pak1) resulting in increased arrhythmic activity. Triggered arrhythmic activity during ischemia is based on changes in cellular ionic balance and the cells Ca²⁺ handling properties. In the current study we used isolated mouse ventricular myocytes (VMs) deficient for the expression of Pak1 (Pak1^{-/-}) to determine the mechanism by which Pak1 influences the generation of arrhythmic activity during simulated ischemia.

The Ca²⁺ transient amplitude and kinetics did not significantly change in wild type (WT) and Pak1^{-/-} VMs during 15 min of simulated ischemia. However, Pak1^{-/-} VMs exhibited an exaggerated increase in [Ca²⁺]_i, which resulted in spontaneous Ca²⁺ release events and waves. The Ca²⁺ overload in Pak1^{-/-} VMs could be suppressed with a reverse mode blocker (KB-R7943) of the sodium calcium exchanger (NCX), a cytoplasmic scavenger of reactive oxygen species (ROS; TEMPOL) or a RAC1 inhibitor (NSC23766). Measurements of the cytoplasmic ROS levels revealed that decreased Pak1 activity in Pak1^{-/-} VMs or VMs treated with the Pak1 inhibitor (IPA3) enhanced cellular ROS production. The Pak1 dependent increase in ROS was attenuated in VMs deficient for NADPH oxidase 2 (NOX2; p47^{phox}^{-/-}) or in VMs where NOX2 was inhibited (gp91ds-tat). Voltage clamp recordings showed increased NCX activity in Pak1^{-/-} VMs that depended on enhanced NOX2 induced ROS production. The exaggerated Ca²⁺ overload in Pak1^{-/-} VMs could be mimicked by low concentrations of ouabain.

Overall our data show that Pak1 is a critical negative regulator of NOX2 dependent ROS production and that a latent ROS dependent stimulation of NCX activity can predispose VMs to

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Ca²⁺ overload under conditions where no significant changes in excitation-contraction coupling are yet evident.

Keywords

p21-activated kinase 1; excitation-contraction coupling; sodium calcium exchanger; ischemia; arrhythmia; cardiomyocyte

Introduction

Ventricular tachyarrhythmia during periods of myocardial infarction or ischemia, are a leading cause of death in patients with coronary artery disease [1]. The basis for these arrhythmic events are ischemia induced modifications of extra- and intracellular ion concentrations as well as changes in cellular metabolism. Reduced cell-cell communication through gap junction channels may further facilitate sustained arrhythmic periods by the formation of reentry circuits [1,2]. Ischemia-induced changes in the ion homeostasis of ventricular myocytes (VMs) include a decreased pH, the accumulation of extracellular potassium ([K⁺]_i) and increased intracellular levels of sodium and calcium ([Na⁺]_i, [Ca²⁺]_i) [3,4]. Na⁺ levels rise due to ATP-depletion and the ROS dependent increase of the late Na⁺-current (I_{NaL}) [5]. The increase in diastolic [Ca²⁺]_i is due to an attenuated Ca²⁺-uptake into the sarcoplasmic reticulum (SR) by the SR Ca-ATPase (SERCA) and enhanced leak of Ca²⁺ from the SR through the ryanodine receptor (RyR). Both effects have been described to depend on ROS-mediated post-translational protein modifications [6].

In different experimental settings increased [Ca²⁺]_i has been linked to an enhanced propensity for triggered arrhythmic activity. The mechanism that translates increased levels of [Ca²⁺]_i into triggered activity is the Na⁺/Ca²⁺-exchanger (NCX) [7]. Due to the electrogenic nature of the transport (1 Ca²⁺: 3 Na⁺) NCX-dependent Ca²⁺ removal from the cytoplasm is linked to a depolarizing inward current which can contribute to a prolongation of the action potential (AP) [8,9]. When SERCA activity is attenuated the enhanced NCX dependent extrusion of Ca²⁺ promotes after depolarizations at the end or in between APs [10,11]. NCX can also operate in the reverse mode, where it contributes to Ca²⁺ entry during the early phase of the AP upstroke [12,13]. While under physiological conditions NCX mediated Ca²⁺ entry is small, changes in [Na⁺]_i and the resting membrane potential (V_m) can potentiate Ca²⁺ entry [14-16]; and, in the context of ischemia reperfusion (I/R) injury contribute to Ca²⁺-overload and spontaneous intracellular Ca²⁺ release events [17,18].

It has recently been demonstrated that the stimulation of p21-activated kinase (Pak1), which is activated by the small Rho GTPases Rac1 and Cdc42, is protective during I/R-injury [19,20]. An improved contractile recovery was attributed to the Pak1-dependent increase in myosin light chain phosphorylation and troponin-T dephosphorylation [20]. A Pak1-dependent anti-arrhythmic action was described to occur through enhanced Akt phosphorylation and concomitant nitric oxide (NO) production by the endothelial nitric oxide synthase (eNOS) [19]. Neither of these studies identified the mechanism by which Pak1 is able to suppress the triggered activity.

We have previously demonstrated that cardiac excitation-contraction coupling (E-CC) is modulated by Pak1 signaling. In VMs stimulation of Pak1 activity maintained the functional interaction between L-type Ca²⁺ channels in the t-tubules and RyRs in the SR [21]. It thereby guarantees the rapid and homogeneous AP-induced increase of the Ca²⁺ transient and maintains its amplitude. Since changes in cardiac E-CC play a prominent role in ischemia-induced arrhythmic activity we aimed to test the hypothesis that under ischemic

conditions Pak1 signaling regulates cellular Ca^{2+} handling properties thereby preventing the induction of arrhythmia. We used isolated VMs from WT and Pak1^{-/-} mice [20-22] that were exposed to simulated ischemia while we monitored excitation-induced Ca^{2+} handling properties [21,23,24]. Our experimental results demonstrate that attenuation of Pak1 signaling increases the cardiomyocytes propensity for Ca^{2+} -overload. The mechanism is based on an increased NADPH oxidase (NOX2) dependent ROS production with a concomitant latent increase in NCX activity under control conditions. The maintenance or stimulation of Pak1 activity could therefore prevent the ROS mediated stimulation of NCX activity that facilitates Ca^{2+} overload and triggered activity during cardiac stress.

Methods

Ventricular myocytes

Ventricular myocytes were isolated from 3-6 month old WT and Pak1 knockout mice (Pak1^{-/-}) by Langendorff perfusion as previously described [21,24]. All animal procedures were performed with the approval of the IACUC of the University of Illinois at Chicago and in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals. Cells were plated on laminin (Sigma Aldrich: 1 mg/ml) coated glass coverslips in standard tyrode solution (in mmol/L: NaCl 130, KCl 5.4, CaCl_2 1, MgCl_2 1.5, NaHCO_3 10, Glucose 10, HEPES 25; pH 7.4) [21,24].

Intracellular Ca^{2+} measurements

To visualize changes of the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), VMs were incubated (15 min) at room temperature with fluo-4 acetoxymethyl ester (5 $\mu\text{mol/L}$; fluo-4/AM; Invitrogen). After washout, 15 min were allowed for de-esterification of the dye. $[\text{Ca}^{2+}]_i$ measurements were performed as previously described [21,24]. Whole-cell Ca^{2+} -transients were recorded by 2D confocal imaging (VTeye; VisiTech International, Sunderland, UK) at sampling frequencies from 120 to 360 Hz [21,24]. The resulting images were analyzed using Metamorph (Molecular Devices) and ImageJ. Ca^{2+} -transients are presented as the change in background-subtracted fluorescence normalized to the diastolic fluorescence (F_0) at the beginning of the recording ($\Delta F/F_0$). VMs were stimulated at twice the threshold at a frequency of 0.5 Hz for the duration of the experiment.

Recordings of intracellular concentrations of reactive oxygen species

To identify reactive oxygen species (ROS) production, isolated VMs were loaded with the sensor 2',7'-dichlorofluorescein diacetate (DCFH) (10 $\mu\text{mol/L}$ for 30 min at 37°C). Baseline fluorescent levels were determined and for time dependent changes the signal was normalized to the fluorescence before stimulation (F_0) and quantified as change in fluorescence over time [24,25]

In vitro model of simulated ischemia reperfusion

To mimic ischemic conditions in vitro VMs were superfused with a solution that mimics hypoxia, hyperkalemia, acidosis, and lactate accumulation [24,26]. The solution contained (mmol/L): NaCl 113, NaHCO_3 6, NaH_2PO_4 0.9, KCl 8, MgCl_2 1.5, CaCl_2 1, Na/lactate 20, (pH 6.8) and was gassed with 95 % N_2 , 5 % CO_2 . Simulated ischemia was maintained for 15 min before reperfusion.

Patch clamp recordings

VMs were used for voltage clamp recordings of the NCX current (I_{NCX}). Experiments were performed in the ruptured patch configuration with microelectrodes filled with pipette solution containing (mmol/L): 110 CsCl, 30 TEA-Cl, 10 NaCl, 10 Hepes, 5 MgATP, pH 7.2

with CsOH. Pipette resistance ranged from 1 M Ω to 3 M Ω . I_{NCX} was recorded as the caffeine (10 mmol/L) induced inward current of voltage clamped VMs clamped at a holding potential of -80 mV. Steady state loading of the SR was assured by conditioning voltage pulses (100 ms) to 0 mV at 1 Hz [27,28]. Cell capacitance was measured during application of -10 mV voltage pulses from a holding potential of -50 mV in the voltage-clamp mode [21].

SDS-PAGE and Immunoblotting

Isolated VMs were lysed directly with the addition of hot 1-X Laemmli sample buffer without β -mercaptoethanol (β -ME) or bromophenol blue dye and heated to 95°C for 5 min. Sample protein determinations were made with a BCA protein assay kit (Pierce) and then β -ME and dye were added to the final concentrations for 1-X sample buffer and heated as before. Cell lysates were separated using pre-cast 4-20 % Novex tris-glycine gels (Invitrogen) and following standard electrophoresis protocols for SDS-PAGE and immunoblotting as previously described [24,29]. Typically 25-45 μ g of protein were loaded per well. Primary antibodies used for Western blotting were directed against the sodium-calcium exchanger (NCX; R&D Systems, PPS019) and α -actinin (Sigma A7811). Species-specific horseradish peroxidase-conjugated secondary antibodies were used and visualization was accomplished using Western Lighting chemi-luminescence reagents (PerkinElmer) and Kodak BioMax film.

Statistics

Data sets were statistically evaluated using an un-paired *t* test. All averaged data are presented as means $\pm$ S.E.M., the number of experiments (n) refers to the number of cells examined and is indicated in the text. For each experimental group cells from at least 2 different cell isolations/animals were used.

Results

Role of Pak1 activity during ischemia/reperfusion

Pak1 was shown to protect cardiomyocytes from I/R-induced arrhythmia [19] and to enhance post-ischemic contractile recovery [20,30]. To determine the mechanism by which Pak1 attenuates ischemia induced triggered activity we exposed isolated WT and Pak1^{-/-} VMs to simulated I/R injury. Changes in $[Ca^{2+}]_i$ were monitored in field-stimulated (0.5 Hz) VMs loaded with the Ca^{2+} sensitive dye Fluo-4/AM. Figure 1 shows representative Ca^{2+} transients recorded in WT (A) and Pak1^{-/-} (B) VMs during pre-ischemia, simulated ischemia (at 2 min and 14 min) and upon reperfusion. As previously shown, under control (pre-ischemic) conditions the Ca^{2+} transient amplitude in Pak1^{-/-} VMs was decreased and the decay constant (τ_{Ca}) was prolonged compared to WT cells [21,31]. Simulated ischemia did not result in a significant change of either the Ca^{2+} transient amplitude or τ_{Ca} in WT or Pak1^{-/-} VMs (Fig. 1C,D) [24]. In both cell types a time-dependent increase in the diastolic $[Ca^{2+}]_i$ was determined. This increase in diastolic $[Ca^{2+}]_i$ was significantly more pronounced in Pak1^{-/-} compared to WT VMs (WT: 1.23 ± 0.08 ; n = 10; Pak1^{-/-}: 1.87 ± 0.12 %; n = 10, 15 min of simulated ischemia, *p* < 0.05; Fig. 1E). The increased diastolic $[Ca^{2+}]_i$ during simulated ischemia was also reflected in an increased Ca^{2+} load of the SR as identified by the amplitude of the caffeine-induced (10 mmol/L) Ca^{2+} transient (Fig. 2AB). While Pak1^{-/-} VMs remained responsive to field stimulation, spontaneous Ca^{2+} release events and waves were frequently determined in 6 out of 6 cells during simulated ischemia (Fig. 1B, arrows). In WT and Pak1^{-/-} VMs Ca^{2+} waves under simulated ischemic conditions exhibited no difference in the Ca^{2+} wave propagation velocity at comparable amplitudes was determined, indicating that RYR receptor open probability was not significantly different between the cells. WT cells remained responsive to field stimulation throughout ischemia

and exhibited the characteristic increase in Ca^{2+} transient amplitude upon reperfusion [24]. In contrast $\text{Pak1}^{-/-}$ VMs upon reperfusion went into contracture, had elevated $[\text{Ca}^{2+}]_i$ and no stimulation-induced Ca^{2+} transients could be induced. The results indicate that loss of Pak1 activity makes VMs prone to ischemia induced Ca^{2+} overload and arrhythmic spontaneous Ca^{2+} release events.

Ca^{2+} -overload in $\text{Pak1}^{-/-}$ VMs depends on increased NCX activity

Over the course of ischemia the decline of the intracellular pH and increase in $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$ can lead to a depolarization of V_m and a shift of the NCX reversal potential [15,17,18]. Concomitantly NCX reverse mode activity is enhanced during the early phase of the action potential (AP) and can increase Ca^{2+} entry into the cell. To determine if in $\text{Pak1}^{-/-}$ VMs the exaggerated Ca^{2+} overload is due to reverse mode NCX activity the pre-ischemic and ischemic solution was supplemented with KB-R7943 (1 $\mu\text{mol/L}$) a reverse mode blocker of NCX [24,32](Fig. 3). KB-R7943 did not significantly change the Ca^{2+} transient amplitude and kinetic in WT or $\text{Pak1}^{-/-}$ VMs when added under pre-ischemic and ischemic conditions (Fig. 3ABC). However, during simulated-ischemia the exaggerated increase in diastolic Ca^{2+} and the associated spontaneous Ca^{2+} release events were now suppressed in $\text{Pak1}^{-/-}$ VMs ($\text{Pak1}^{-/-}$: pre-I: 1.02 ± 0.02 ; I_{+KB-R} : 1.13 ± 0.07 ; $n = 4$; Fig. 3D). This result indicates that NCX mediates the ischemia induced Ca^{2+} overload in $\text{Pak1}^{-/-}$ VMs.

To determine if NCX activity is increased in $\text{Pak1}^{-/-}$ VMs, we recorded I_{NCX} during whole cell voltage clamp recordings as the caffeine-induced (10 mmol/L) inward current at a holding potential of -80 mV [27,28]. Under pre-ischemic conditions where SR load is comparable in WT and $\text{Pak1}^{-/-}$ VMs (Fig. 2B; [21,33]). The peak amplitude of I_{NCX} (WT: 267.2 ± 41 pA, $n = 7$; $\text{Pak1}^{-/-}$: 409.5 ± 75 pA; $n = 10$; Fig. 4A) and the current density (WT: 1.08 ± 0.1 pA/pF, $n = 7$; $\text{Pak1}^{-/-}$: 2.28 ± 0.31 pA/pF, $n = 10$, $p < 0.05$; Fig. 4B) were both increased in $\text{Pak1}^{-/-}$ VMs. Cell capacitance in $\text{Pak1}^{-/-}$ VMs is decreased [21,34,35] but plotting I_{NCX} as a function of cell capacitance further confirmed that I_{NCX} was increased in $\text{Pak1}^{-/-}$ compared to WT VMs over the entire range of capacitances (Fig. 4C). The increase in I_{NCX} was not due to increased NCX protein levels in $\text{Pak1}^{-/-}$ compared to WT VMs as determined by western blotting (Fig. 4D). The results indicate that in the absence of Pak1, NCX activity is latently increased under control conditions, which makes the cells susceptible to Ca^{2+} overload during ischemic stress.

NCX activity depends on ROS production

It has been described that ROS-dependent NCX activation plays a role in I/R-induced Ca^{2+} overload [36]. To determine if NCX activity in $\text{Pak1}^{-/-}$ VMs is already enhanced in a ROS-dependent manner under pre-ischemic conditions, we quantified NCX activity in the presence and absence of the ROS scavenger TEMPOL (TEMP: 100 $\mu\text{mol/L}$). Caffeine-induced Ca^{2+} transients were initiated in WT and $\text{Pak1}^{-/-}$ VMs and the caffeine transient decay constant (τ_{caff}) was quantified as a measure of NCX activity (Fig. 5AB). As previously described the caffeine transient amplitude in WT and $\text{Pak1}^{-/-}$ VMs was comparable (Fig. 5C, [21]) under control conditions however, τ_{caff} was significantly accelerated in $\text{Pak1}^{-/-}$ VMs (WT: 2.87 ± 0.15 s, $n = 33$; $\text{Pak1}^{-/-}$: 2.32 ± 0.24 s; $n = 7$, $p < 0.05$; Fig. 5AB). In the presence of TEMPOL, τ_{caff} increased in $\text{Pak1}^{-/-}$ VMs and became comparable to WT cells (WT_{TEMP}: 3.0 ± 0.18 s, $n = 9$; $\text{Pak1}^{-/-}TEMP: τ_{caff} 2.75 ± 0.17 s; $n = 12$). No change in the caffeine transient amplitude was determined during TEMPOL superfusion (Fig. 5C). The experiments indicate that in $\text{Pak1}^{-/-}$ VMs under pre-ischemic conditions NCX activity is already enhanced due to an apparent increase in the basal ROS production.$

Pak1 regulates basal ROS production through NOX2

To identify the levels of ROS production in WT and Pak1^{-/-} VMs we monitored basal ROS production in VMs loaded with the ROS sensor DCFH (10 μ mol/L for 30 min at 37°C). In freshly isolated VMs the basal DCF fluorescence was significantly increased in Pak1^{-/-} VMs compared to WT cells (WT: 548 ± 164 a.u., $n = 22$; Pak1^{-/-}: 7440 ± 1557 a.u.; $n = 15$; Fig. 6AB). To identify if the change in ROS depended directly on the loss of Pak1 activity, VMs were treated with the Pak1 inhibitor 2,2'-Dihydroxy-1,1'-dinaphthylidylsulfide (IPA3: 10 μ mol/L [25,37]). After 2 h of incubation, WT myocytes exhibited increased basal ROS levels while in Pak1^{-/-} VMs no IPA3-dependent change in fluorescence was determined (WT_{IPA3}: 5236 ± 829 a.u., $n = 15$; Pak1^{-/-}_{IPA3}: 6648 ± 1386 a.u.; $n = 15$; Fig. 6AB). In non-cardiomyocytes a Pak1-dependent regulation of the NADPH-oxidase 2 (NOX2) was previously described [34]. To determine if NOX2 is the primary source of ROS in Pak1^{-/-} cells, we determined ROS levels in VMs deficient of NOX2 activity (p47^{phox}^{-/-} mice [3]) in the presence and absence of IPA3. Under basal conditions ROS levels in NOX2^{-/-} VMs were comparable to WT cells; however, IPA3-dependent ROS production was significantly attenuated (p47^{phox}^{-/-}: 654 ± 60 a.u., $n = 30$; p47^{phox}^{-/-}_{IPA3}: 1495 ± 176 a.u.; $n = 27$; Fig. 6B). To determine the role of NOX2 in basal ROS generation in WT and Pak1^{-/-} VMs NOX2 activity was inhibited by superfusion with the NOX2 inhibiting peptide gp91ds-tat (1 μ mol/L) or by inhibiting Rac1 (NSC23766: 20 μ mol/L) whose interaction with p67^{phox} is a prerequisite of NOX2 activation. While neither of the inhibitors affected basal ROS levels in WT VMs, basal ROS in Pak1^{-/-} VMs was significantly suppressed. To identify the effect of Pak1 inhibition on the rate of ROS production the change in DCF fluorescence was monitored over time in WT cells during superfusion with IPA-3 and subsequent NOX2 inhibition by gp91ds-tat (Fig. 6C). The gp91ds-tat scrambled peptide (1 μ mol/L) was used as a control. The experiments revealed that the rate of ROS production significantly increased upon Pak1 inhibition. gp91ds-tat was able to attenuate the IPA-3 induced increase in slope while the scrambled peptide remained without effect (Fig. 6D). The experiments support that NOX2 is the source of enhanced ROS production when Pak1 activity is attenuated. To determine if the NOX2 derived ROS is the mediator of enhanced NCX activity, WT and Pak1^{-/-} VMs were superfused with the NOX inhibitor apocynin (200 μ mol/L). Apocynin did not induce a change in the caffeine transient amplitude (Fig. 5C). However, τ_{caff} was significantly prolonged in Pak1^{-/-} VMs making it comparable to WT VMs (Pak1^{-/-}_{+Apo}: τ_{caff} : 2.82 ± 0.18 s; $n = 8$; Fig. 5BC).

To test they hypothesis if the increased basal ROS production is sufficient to promote the exaggerated Ca²⁺ overload in Pak1^{-/-} VMs we monitored the rate of ROS production in WT and Pak1^{-/-} VMs under pre-ischemic and simulated-ischemic conditions as well as during reperfusion (Fig. 7A). Simulated ischemia did not significantly enhance ROS production in WT and Pak1^{-/-} VMs, which is reflected in the increase in DCF fluorescence over time (Fig. 7B). Only upon reperfusion a sudden surge in ROS was determined. The result indicates that no additional ROS production occurs during the simulated ischemia. To identify if the increased pre-ischemic level of NOX2/ROS is the reason for the facilitated NCX dependent Ca²⁺ overload during simulated-ischemia, WT and Pak1^{-/-} VMs were exposed to simulated ischemia in the presence of the ROS scavenger TEMPOL (100 μ mol/L), the Rac1 inhibitor NSC23766 (20 μ mol/L) or the NOX inhibitor apocynin (Apo: 200 μ mol/L). During all treatments the excessive Ca²⁺ overload in in WT and Pak1^{-/-} (Fig. 7CD) VMs was suppressed ($\Delta F/F_0$: Pak1^{-/-}: 1.9 ± 0.1 ; $n = 8$; Pak1^{-/-}_{+TEMPOL}: 1.24 ± 0.04 ; $n = 8$; Pak1^{-/-}_{+NCS}: 1.16 ± 0.06 ; $n = 3$; Pak1^{-/-}_{+Apo}: 1.19 ± 0.05 ; $n = 4$; Fig. 7D).

Besides an increase in ROS production an exaggerated Ca²⁺ overload during simulated ischemia could also be the consequence of a pH dependent Na⁺ deregulation. To rule this out we chose an alternative experimental approach to unmask a latent increase in NCX activity. Inhibition of the Na⁺/K⁺-ATPase (NKA) in WT and Pak1^{-/-} VMs with low doses of

ouabain (10 $\mu\text{mol/L}$) leads to a ROS and pH independent increase in $[\text{Na}^+]_i$ and subsequently $[\text{Ca}^{2+}]_i$. As reported previously, ouabain increased the Ca^{2+} transient amplitude and attenuated τ_{Ca} (Fig. 8ACD) in a time dependent manner. However, in $\text{Pak1}^{-/-}$ VMs, ouabain resulted in an exaggerated increase in diastolic Ca^{2+} and spontaneous Ca^{2+} release events and waves. In WT cells ouabain over 20 min at 10 $\mu\text{mol/L}$ did not significantly increase diastolic $[\text{Ca}^{2+}]_i$. Overall these data further support that loss of Pak1 activity predisposes VMs to Ca^{2+} overload and arrhythmia through a latent increase in NCX activity.

Discussion

In the current study we present evidence that in ventricular myocytes attenuated Pak1 signaling increases NCX activity independent of changes in protein expression. The increased NCX activity is sensitive to ROS scavengers and depends on the activity of NOX2. The changes that are latently present under control conditions make VMs prone to NCX-dependent Ca^{2+} -overload independent of an additional increase in ROS.

Pak1 dependent regulation of $[\text{Ca}^{2+}]_i$ in ventricular myocytes

Previous studies reported that after I/R injury Pak1 activity decreased together with a decrease in Akt phosphorylation [19]. In this setting the restoration of Pak1 signaling with the sphingosine-1-phosphate derivative FTY720 attenuated the arrhythmic activity in isolated Langendorff-perfused hearts. The mechanism of enhanced triggered activity was not identified in these experiments.

Our experimental results show that attenuated Pak1 activity results in spontaneous Ca^{2+} release events and waves during simulated ischemia as well as during NKA inhibition (Fig. 1 & 8). An increase in spontaneous Ca^{2+} release events and waves is often associated with an increased open probability of RYR [6]. This can be enhanced through CaMKII dependent phosphorylation of RYR and/or oxidative protein modifications [38,39]. While an enhanced open probability of RYR is consistent with the spontaneous release events seen in the $\text{Pak1}^{-/-}$ VMs, increased RYR leak is generally associated with a decrease in SR load [40] which is not consistent with the increased SR load in $\text{Pak1}^{-/-}$ VMs during simulated ischemia (Fig. 2A). Also the Ca^{2+} wave propagation velocity in $\text{Pak1}^{-/-}$ VMs at comparable wave amplitude is not different from WT cells and therefore does not support facilitated Ca^{2+} release from RYR [41]. The mutant R4496C enhances RYR open probability in VMs that exhibit increased spontaneous release events compared to WT VMs under control conditions and in response to ouabain superfusion [42]. However, in contrast to $\text{Pak1}^{-/-}$ VMs these events are independent of an exaggerated increase diastolic $[\text{Ca}^{2+}]_i$ or SR load. We therefore propose that the spontaneous release events induced by simulated ischemia or ouabain in $\text{Pak1}^{-/-}$ VMs are independent of changes in RYR open probability.

Spontaneous release events can further be the consequence of Ca^{2+} overload as a consequence of increased Ca^{2+} entry or decreased Ca^{2+} extrusion from the cell. The L-type Ca^{2+} channel (LTCC, $\text{Ca}_v1.2$) is the dominating source of Ca^{2+} entry in VMs and LTCC current ($I_{\text{Ca,L}}$) as well as protein levels were shown to be comparable between WT and $\text{Pak1}^{-/-}$ VMs [21]. During simulated ischemia a decrease in LTCC current was described but changes were not significant up to 15 min of exposure [26]. Increased LTCC dependent Ca^{2+} entry is therefore not likely to be the reason for Ca^{2+} overload in $\text{Pak1}^{-/-}$ VMs. While in the presence of ouabain an increase in LTCC dependent Ca^{2+} entry was described, inhibition of LTCC in these conditions only accounted for a 30% reduction in Ca^{2+} supporting that LTCC is not the dominating mechanism of Ca^{2+} overload [43].

Cytosolic Ca^{2+} can further increase due to enhanced SERCA activity or decreased NCX dependent extrusion of Ca^{2+} to the extracellular space. The increased SR load in $\text{Pak1}^{-/-}$ VMs during simulated ischemia would support this hypothesis; however, our experimental data do not demonstrate a facilitated SR Ca release under these conditions (Fig. 1D). While we report that NCX activity is increased in $\text{Pak1}^{-/-}$ VMs this is not reflected in a faster Ca^{2+} transient decay constant. But in mice the Ca^{2+} transient decay constant reflects to 90% the SERCA dependent Ca^{2+} re-uptake into the SR. Consequently our results would support that there is no difference in SERCA activity between WT and $\text{Pak1}^{-/-}$ VMs.

An alternative way of Ca^{2+} entry is the reverse mode of NCX [9]. Although the window of opportunity is small, I_{NCX} was shown to modulate the Ca^{2+} transient amplitude under control conditions [13,16]. During ischemia this window of opportunity increases when $[\text{Na}^+]_i$ increases and the membrane potential depolarizes [14,36]. The increased I_{NCX} in $\text{Pak1}^{-/-}$ VMs under control conditions and suppression of Ca^{2+} overload during simulated ischemia in the presence of the NCX blocker KB-R7943 support NCX as the source of enhanced Ca^{2+} entry under these conditions (Fig. 3 & 4). Enhanced NCX activity in $\text{Pak1}^{-/-}$ VMs could also be a reason for the observed AP prolongation and would be consistent with the exaggerated Ca^{2+} overload in the presence of ouabain. Ouabain inhibits NKA and leads to an accumulation of $[\text{Na}^+]_i$ [42]. Even if the rate of Na^+ accumulation is comparable between WT and $\text{Pak1}^{-/-}$ VMs, increased NCX activity would accelerate the rate of Ca^{2+} entry in the $\text{Pak1}^{-/-}$ VMs.

As indicated, Ca^{2+} entry through the reverse mode of NCX depends critically on $[\text{Na}]_i$ [16]. Simulated ischemia favors the accumulation of $[\text{Na}^+]_i$ through increased Na^+/H^+ -exchanger activity [14], a ROS/CaMKII-dependent increase in the late- Na^+ current ($I_{\text{Na,L}}$) [8,10] and attenuated Na^+ removal through the Na^+/K^+ -ATPase. The enhanced NCX reverse mode activity in $\text{Pak1}^{-/-}$ VMs could therefore be alternatively explained by an enhanced Na^+ accumulation in these cells. NKA protein levels are comparable between WT and $\text{Pak1}^{-/-}$ VMs (not shown) and the effect of ouabain on $\text{Pak1}^{-/-}$ Ca^{2+} handling properties (Fig. 8) supports that NKA activity is not suppressed under control conditions. The fact that Ca^{2+} overload in $\text{Pak1}^{-/-}$ VMs is exaggerated during simulated ischemia as well as ouabain, further excludes an enhanced Na^+ accumulation in $\text{Pak1}^{-/-}$ VMs due to pH deregulation [14]. In yet another scenario $[\text{Na}^+]_i$ could be enhanced in $\text{Pak1}^{-/-}$ VMs due to a ROS dependent increase in $I_{\text{Na,L}}$ [8,10]. This hypothesis would be consistent with the prolonged AP duration $\text{Pak1}^{-/-}$ VMs [21]. At this point we cannot rule out that $I_{\text{Na,L}}$ in $\text{Pak1}^{-/-}$ VMs is increased; however, this would mean that $[\text{Na}^+]_i$ is already increased under control conditions. Increased $[\text{Na}^+]_i$ would enhance NCX reverse mode while it would rather attenuate NCX dependent Ca^{2+} removal. The latter is inconsistent with our experiments that clearly demonstrate an enhanced NCX-dependent Ca^{2+} extrusion in $\text{Pak1}^{-/-}$ VMs. We therefore propose that increased NCX activity is sufficient to make $\text{Pak1}^{-/-}$ VMs susceptible to Ca^{2+} overload under conditions of stress.

ROS-dependent regulation of NCX

Our experiments indicate that $\text{Pak1}^{-/-}$ VMs have an increased susceptibility to Ca^{2+} overload under conditions of cellular stress (in our cases: simulated ischemia and NKA inhibition). The Ca^{2+} overload can be based on stress-induced changes in the Ca^{2+} handling properties of $\text{Pak1}^{-/-}$ VMs or latent changes in the Ca^{2+} handling properties of $\text{Pak1}^{-/-}$ VMs that only become apparent during increased stress.

We demonstrated that $\text{Pak1}^{-/-}$ VMs exhibit increased NCX activity under control conditions that is sensitive to ROS scavengers. The data are consistent with increased basal ROS levels in $\text{Pak1}^{-/-}$ VMs and therefore suggest that changes in Ca^{2+} handling are already present under control conditions. This is further supported by the fact that simulated-ischemia and

ouabain have the same effect and occur even though simulated-ischemia does not further increase the basal rate of ROS production (Fig. 7AB). Increased levels of ROS, through superfusion of VMs with H₂O₂ or xanthine and xanthine oxidase resulted in increased NCX inward as well as reverse mode activity [44,45]. ROS-dependent thiol-modifications of the NCX protein [30], but also a regulation through activated p44-MAPK have been suggested [31]. The suppression of NCX-dependent Ca²⁺ overload as well as the increase of τ_{caff} in the presence of ROS scavengers supports a ROS dependent regulation of NCX in Pak1^{-/-} VMs. Interestingly, ROS not only regulates NCX but was also described as a potent regulator of E-CC proteins such as RYR and SERCA [46]. In experiments on isolated VMs superfused with H₂O₂ it was suggested that the ROS induced increase in [Ca²⁺]_i is predominantly due to increased SR Ca²⁺ release [47]. NCX under these experimental conditions did not play a prominent role. In our model NCX seems to be the more sensitive read out for NOX2-dependent ROS production and an increase in NCX activity was detectable even before changes in RYR dependent Ca²⁺ leak were determined. One explanation for this differential finding could be that under control conditions especially in mouse myocytes NCX activity only has a small contribution to the Ca²⁺ transient amplitude and decay. As a consequence an increased activity of NCX may remain undetected in the absence of an increase in [Na⁺]_i [13,16] which again would depend on the length and concentration of H₂O₂ exposure.

Source of ROS production in Pak1^{-/-} VMs

NADPH oxidases are cytoplasmic sources of ROS production in cardiomyocytes. The NOX isoforms 2 and 4 are predominantly expressed in heart where NOX2 produces ROS in response to agonist induced stimulation of G-protein coupled receptors (G_i, G_{12/13}, G_q) [33]. Under physiological conditions the activation of NOX2 depends on the interaction of the membrane components gp91^{phox} and p22^{phox} with a hetero-multimeric cytoplasmic protein complex (p47^{phox}, p40^{phox}, p67^{phox}) [33]. The membrane translocation of the cytoplasmic complex requires Rac1 binding and p47^{phox} phosphorylation. p47^{phox} was described as a target of Pak1 phosphorylation and in neutrophils Pak1 activity was required for NOX-dependent ROS production [34,35]. Under our experimental conditions decreased Pak1 activity (Pak1^{-/-} or IPA3) enhanced ROS production (Fig. 6) The inhibitory effect of the peptide gp91ds-tat and the NSC 23766 (Fig. 6BCD) support NOX2 as the source of these increased basal ROS levels. Pak1^{-/-} and WT VMs maintained their rate of ROS production during simulated ischemia. This is consistent with previous reports that the major increase in ROS occurs upon reperfusion through the mitochondrial electron transport chain [48]. We do not exclude that other sources of ROS could enhance NCX activity, but the sole modification of NCX by NOX2 could suggest a preferential interaction that is favored by a sub-cellular co-localization. While we have ruled out changes in protein expression of gp91^{phox}, p47^{phox} as well as p47^{phox} phosphorylation (not shown), at this point we have not identified the mechanism by which Pak1 negatively regulates NOX2 activity. One possibility, is a role of Pak1 in the assembly and disassembly of the hetero-multimeric protein complex of NOX2 [25], which has been demonstrated to be critically dependent on cytoskeletal remodeling [25].

Conclusion

During ischemia decreased Pak1 activity was shown to result in a QT prolongation, an increased number of ectopic beats and ventricular tachycardia Langendorff perfused hearts [19]. We present experimental evidence that attenuation of Pak1 activity enhances baseline NOX2-dependent ROS production and for the first time demonstrate that NCX activity is a sensitive read out mechanism for NOX2 derived ROS. Under physiological conditions the NOX2/ROS mediated increase in NCX activity does not significantly impact beat-to-beat

changes in $[Ca^{2+}]_i$; however, the latent increase in NCX activity makes ventricular myocytes susceptible for Ca^{2+} overload and arrhythmic Ca^{2+} release during increased $[Na^+]_i$. The results are consistent with previous studies in Langendorff perfused hearts and would support the NCX-mediated Ca^{2+} overload as the trigger for ectopic beats and ventricular tachycardia. This study indicates that stimulation of Pak1 activity could represent a mechanism through which to prevent NCX dependent Ca^{2+} overload in the stressed or arrhythmic myocardium.

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Highlights

- Loss 1 of Pak1 activity increases basal ROS levels
- Pak1 is a negative regulator of NOX2 in cardiomyocytes
- NOX2 derived ROS increases NCX activity
- latent increase in NCX activity predisposes cardiomyocytes to Ca²⁺ overload

Figure 1. Ischemia/reperfusion induced changes in Ca-handling properties

Representative whole cell field stimulation induced Ca transients recorded in WT (A) and *Pak1*^{-/-} (B) VM during pre-ischemia (pre-I), ischemia (I) and reperfusion. Arrows mark spontaneous Ca release events independent from field stimulation. Bar graphs show (C) the Ca transient amplitude, (D) the Ca transient decay constant (τ_{Ca}) and (E) the change in diastolic Ca in WT (black) and *Pak1*^{-/-} (grey) VMs under pre-ischemic (pre-I) conditions and after 14 min of ischemia (I: hatched bars). (F) (*: $p < 0.05$ compared to pre-I; &: $p < 0.05$ compared to WT).

Figure 2. Ischemia induced changes in SR load and spontaneous Ca release

Simulated ischemia induced changes in diastolic Ca (**A**) and caffeine (10 mmol/L; **B**) transient amplitude in WT and Pak1^{-/-} VMs. Spontaneous Ca waves during simulated ischemia have comparable amplitude (**C**) and conduction velocity (**D**) in WT and Pak1^{-/-} VMs. (*: $p < 0.05$ compared to pre-I)

Figure 3. NCX reverse mode blocker prevents Ca overload during simulated ischemia
 Representative field stimulation induced Ca transients recorded in WT (**A, top**) and Pak1^{-/-} (**bottom**) VM during pre-ischemia, simulated ischemia (I) and reperfusion in the absence and presence of KB-R7943 (1 μ mol/L). Bar graphs show (**B**) the Ca transient amplitude, (**C**) the Ca transient decay constant (τ_{Ca}) and (**D**) the change in diastolic Ca in WT (black) and Pak1^{-/-} (grey) VMs under pre-ischemic (pre-I) conditions and after 14 min of simulated ischemia (I: hatched bars).

Figure 4. NCX inward current is increased in $Pak1^{-/-}$ VMs

(A) The mean I_{NCX} current amplitude and (B) I_{NCX} current density is shown that was recorded in response to 10 mmol/L caffeine in WT (black; $n = 7$) and $Pak1^{-/-}$ (grey; $n = 10$) VMs. (C) The plot of I_{NCX} against cell capacitance shows that I_{NCX} is increased independent of cell capacitance. (D) Western blot showing that NCX expression levels are unchanged in $Pak1^{-/-}$ VMs; α -actinin is shown as a loading control. (*: $p < 0.05$ compared to WT).

Figure 5. Enhanced NCX dependent Ca removal in Pak1^{-/-} myocytes depends on ROS
 (A) Representative stimulation and caffeine (10 mmol/L) induced Ca transients in WT (black) and Pak1^{-/-} (grey) VMs. (B) The caffeine transient decay constant (τ_{caff}) and caffeine transient amplitude (C) recorded in WT (black; n = 33) and Pak1^{-/-} (grey; n = 7) VMs during superfusion with either TEMPOL (100 μ mol/L; n = 12) or apocynin (200 μ mol/L; n = 8) (*: p < 0.05 compared to WT).

Figure 6. Attenuated Pak1 activity increases NOX2-dependent ROS production

(A) Representative images of WT (top) and Pak1^{-/-} (bottom) VMs loaded with DCF in the absence (left) and presence (right) of the Pak1 inhibitor IPA-3 (10 μmol/L). (B) Bar graph representing DCF fluorescence (mean ± SEM) in WT (black), Pak1^{-/-} (grey) and p47^{phox}^{-/-} VMs (white) in the presence and absence of IPA-3, the Rac1 inhibitor NSC23766 (20 μmol/L) or the NOX2 specific inhibitor gp91ds-tat (1 μmol/L). (C) Time dependent change in DCF fluorescence during subsequent superfusion of WT cells with IPA-1 and gp91ds-tat or the scrambled control peptide (1 μmol/L). (D) Bar graph demonstrating the rate of fluorescence-increase in WT cells during Ctrl, IPA-3, IPA-3 + scrambled peptide and IPA-3 plus gp91ds-tat. (*: p < 0.05 compared to WT).

Figure 7. Exaggerated Ca overload during simulated ischemia in Pak1^{-/-} VMs depends on baseline NOX2 activity

(A) Time dependent change in DCF fluorescence in WT and Pak1^{-/-} VMs during pre-ischemic conditions, simulated ischemia and reperfusion. (B) Bar graphs illustrate that the rate of ROS production is increased in Pak1^{-/-} VMs, the rate remains unchanged in WT and Pak1^{-/-} VMs during simulated ischemia. Average change in diastolic Ca²⁺ in WT (C) and Pak1^{-/-} (D) VMs during pre-ischemic conditions (pre-I) and simulated ischemia (I) in the absence and presence of TEMPOL (100 μmol/L; n = 8), the Rac1 inhibitor NSC23766 (20 μmol/L) or apocynin (Apo: 200 μmol/L; n = 4). (*: p < 0.05)

Figure 8. Ouabain induced exaggerated Ca^{2+} overload in Pak1^{-/-} VMs

Representative Ca^{2+} transient from WT (A, top) and Pak1^{-/-} (bottom) VMs during superfusion with ouabain (10 μ mol/L). (B) Time dependent change in diastolic Ca^{2+} in WT and Pak1^{-/-} VMs during ouabain superfusion. Ouabain induced changes in (C) Ca^{2+} transient amplitude, (D) τ_{Ca} and (E) diastolic Ca^{2+} after 20 min of ouabain superfusion. (*: $p < 0.05$)