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Pak1 Is Required to Maintain Ventricular Ca²⁺ Homeostasis and Electrophysiological Stability through SERCA2a Regulation in Mice

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Abstract

Background—Impaired sarcoplasmic reticular (SR) Ca²⁺ uptake resulting from decreased SR Ca²⁺-ATPase type 2a (SERCA2a) expression or activity is characteristic of heart failure (HF) with its associated ventricular arrhythmias. Recent attempts at gene therapy of these conditions explored strategies enhancing *SERCA2a* expression and/or activity as novel approaches to HF management. We here explore the role of Pak1 in maintaining ventricular Ca²⁺ homeostasis and electrophysiological stability under both normal physiological, and acute and chronic β -adrenergic stress conditions.

Methods and Results—Mice with a cardiomyocyte-specific *Pak1* deletion (*Pak1*^{cko}), but not controls (*Pak1*^{f/f}), showed high incidences of ventricular arrhythmias and electrophysiological instability during either acute β -adrenergic or chronic β -adrenergic stress leading to hypertrophy, induced by isoproterenol. Isolated *Pak1*^{cko} ventricular myocytes correspondingly showed aberrant cellular Ca²⁺ homeostasis. *Pak1*^{cko} hearts showed an associated impairment of SERCA2a function

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and down-regulation of SERCA2a mRNA and protein expression. Further explorations of the mechanisms underlying the altered transcriptional regulation demonstrated that exposure to control *Ad-shC2* virus infection increased SERCA2a protein and mRNA levels following phenylephrine stress in cultured neonatal rat cardiomyocytes (NRCMs). This was abolished by the Pak1-knockdown in *Ad-shPak1*-infected NRCMs and increased by constitutive over-expression of active Pak1 (*Ad-CAPak1*). We then implicated activation of serum response factor (SRF), a transcriptional factor well-known for its vital role in regulation of cardiogenesis genes in the Pak1-dependent regulation of SERCA2a.

Conclusions—These findings indicate that Pak1 is required to maintain ventricular Ca^{2+} homeostasis and electrophysiological stability and implicate Pak1 as a novel regulator of cardiac SERCA2a through a transcriptional mechanism.

Keywords

cardiac arrhythmia; calcium signaling; sarcoplasmic reticulum Ca^{2+} -ATPase; P21 activated kinase 1

Introduction

Sudden arrhythmic death (SAD), a major public health challenge, often occurs with the contractile dysfunction and electrophysiological instability associated with cardiac hypertrophy and heart failure (HF). Its mechanisms remain elusive, compromising development of effective anti-arrhythmic therapies. Nevertheless, defective regulation of Ca^{2+} cycling proteins including ryanodine receptor type 2 (RyR2), RyR2- Ca^{2+} release channel macromolecular complexes and sarcoplasmic reticular (SR) Ca^{2+} -ATPase type 2a (SERCA2a)/phospholamban (PLN) complexes may contribute to its pathophysiology and progression¹.

Gene therapy seeking enhanced SERCA2a expression and/or activity to restore contractile function and electrophysiological stability in both HF patients and animal models over the past decade prompted the CUPID trial in the USA exploring intracoronary delivery of adeno-associated virus (AAV) expressing SERCA2a in HF patients^{2, 3}; a multi-centre SERCA2a gene therapy program has recently commenced in the UK. Although increased SR Ca^{2+} uptake might also increase SR Ca^{2+} leak potentially triggering ventricular activity, further studies confirmed that SERCA2a gene transfers tend to restore electrophysiological stability offering potential anti-arrhythmic strategies. However, gene therapy may be clinically limited by its short duration of action, immune and inflammatory responses, uncertainties in specifically targeting particular tissues, and difficulties in gene dosage control. Finally, pre-existing anti-AAV1 neutralizing antibodies against viral capsid proteins may inhibit vector uptake in patients previously exposed to AAV⁴. These difficulties prompted other possible therapeutic strategies for modulating SERCA2a activity including targeting its SUMOylation⁵.

Recent work has identified and characterized cardiac, including anti-hypertrophic, effects of the multifunctional p21-activated kinase 1 (Pak1), abundant in heart⁶⁻⁸. We now demonstrate that Pak1 is required to maintain ventricular Ca^{2+} homeostasis and

electrophysiological stability and this effect is largely mediated through its regulation of *SERCA2a* expression involving a transcriptional mechanism. Mouse hearts with cardiomyocyte-specific *Pak1* deletion (*Pak1^{cko}*) showed arrhythmic instabilities particularly following either acute or chronic β -adrenergic stress. This accompanied aberrant cellular Ca^{2+} homeostasis attributable to down-regulated SERCA2a function mediated through the widely expressed, transcription factor, serum response factor (SRF) known to be vital in gene regulation during cardiogenesis^{9,10}.

Methods

Animals

Homozygous *Pak1* flox (*Pak1^{f/f}*) mice were used in control groups against mice expressing cardiac-specific knockout of *Pak1* generated using the Cre-LoxP system whereby Cre is expressed under the α -myosin heavy chain promoter (further details described by Liu *et al*, 2011¹). 8-10 week old male *Pak1^{f/f}* and *Pak1^{cko}* mice were subjected to either isoprenaline (ISO, Sigma) at 10 mg/kg/day to induce chronic β -adrenergic, hypertrophic stress conditions or vehicle (saline) for 14 days via subcutaneously implanted osmotic mini-pumps (Alzet). Animal studies including all procedures were performed in accordance with UK Home Office and institutional guidelines.

Detailed Methods for *in vivo*, *ex vivo* and single cell electrophysiological measurements, single cell $[\text{Ca}^{2+}]_i$ measurements, molecular biological experiments are described in the online-only Data Supplement. Animal studies including all procedures were performed in accordance with UK Home Office and institutional guidelines.

Data Analysis and Statistical Methods

In the analysis of the continuous experimental data, values are reported as means $\pm$ SEMs of *n* experiments, and the observations were thought to be sufficiently close to a 'normal' distribution, as judged by Kolmogorov-Smirnov Normality, to permit use parametric tests. Paired or unpaired *t* tests were used as appropriate. Comparisons of more than one factor or between multiple groups utilised two-way ANOVA, corrected by Bonferroni post hoc *t* tests if necessary. The analysis of categorical data employed Fisher's exact tests. In the statistical tests significance was taken to correspond to a probability 0.05. All p-values are two-sided, the statistical software used to generate the results are SPSS and PRISM software.

Results

Pak1 deficiency increases ventricular tachyarrhythmic susceptibility in intact hearts

Anaesthetized *Pak1^{cko}* and *Pak1^{f/f}* mice showed similar baseline electrocardiographic P wave durations and P-R, QRS and QT intervals (Fig.1A,B) but *Pak1^{cko}* mice showed higher heart rates (HR) (Fig.1B). Occasional *Pak1^{cko}* (2 out of 25) but no *Pak1^{f/f}* mice (0 out of 25) showed frequent ventricular ectopics (Fig.1A). Moreover, *Pak1^{cko}*, but not *Pak1^{f/f}* mice had high incidences of ventricular tachyarrhythmias (Fig.1C, D: ventricular tachycardia (VT) and ventricular fibrillation (VF)). These changes occurred following ventricular programmed electrical stimulation (PES) with S1S1 burst pacing, but not S1S2 extra-

stimulus pacing, particularly at higher pacing frequencies, and following acute isoproterenol (ISO) administration (basal cycle length (BCL)<50 ms).

Isolated Langendorff-perfused *Pak1^{cko}* (Fig.1Eii, Eiv) correspondingly demonstrated increased ventricular tachyarrhythmic incidences compared to *Pak1^{f/f}* hearts (Fig.1Ei, Eiii) whether during spontaneous activity (Fig.1Ei, Eii) or before and following burst pacing after acute ISO challenge (Fig.1Eiii, Eiv) during monophasic action potential (MAP) recording. Larger number of *Pak1^{cko}* than *Pak1^{f/f}* hearts showed alternans (Fig.1Fi, ii) and multiple polymorphic VT episodes leading to VF (Fig.1Fiii, Fiv) with chronic β -adrenergic stress induced by chronic ISO treatment (10 mg/kg/day for 14 days) administered using osmotic-mini-pumps (Fig.1G). Thus although *Pak1^{cko}* hearts showed incidences (1 of 14) of VT/VF indistinguishable from *Pak1^{f/f}* hearts (1 of 12) under baseline conditions, *Pak1^{cko}* hearts showed higher VT/VF incidences than *Pak1^{f/f}* hearts under both acute and chronic β -adrenergic stress conditions. Nevertheless, *Pak1^{f/f}* and *Pak1^{cko}* hearts showed indistinguishable ventricular effective refractory periods (VERPs) whether in the absence or presence of either acute or chronic β -adrenergic stress conditions (Fig.1H). Pak1 is thus critical to ventricular electrical stability under conditions of either acute and chronic β -adrenergic stress induced by acute or chronic administration of ISO.

Pak1 deficiency produces instabilities in Ca^{2+} handling

Isolated *Pak1^{cko}* and *Pak1^{f/f}* ventricular myocytes demonstrated significant differences in Ca^{2+} homeostasis. Fig.2A compares Ca^{2+} transients from *Pak1^{f/f}* and *Pak1^{cko}* myocytes during 0.5, 1 and 3 Hz field stimulation under baseline or chronic β -adrenergic stress conditions. *Pak1^{cko}* myocytes showed higher occurrences of arrhythmic-like Ca^{2+} events (irregular, alternating Ca^{2+} transients and/or Ca^{2+} waves) with increased stimulation frequencies than *Pak1^{f/f}* myocytes under both baseline and chronic β -adrenergic stress conditions (Fig.2B).

These instabilities correlated with differences in evoked cytosolic and SR Ca^{2+} responses (Fig.3) among *Pak1^{f/f}* (blue traces) and *Pak1^{cko}* myocytes (red traces) from mice reared under either baseline (Fig.3A-D: left panels) or chronic β -adrenergic stress conditions (Fig. 3A-D: right panels):

1. Time constants for decays of I_{NCX} following its induction by 10 mM caffeine (Fig. 3Ai) were similar in *Pak1^{f/f}* and *Pak1^{cko}* myocytes under baseline conditions but significantly greater in *Pak1^{cko}* ($5.95 \pm 0.20\text{s}$, $n=11$) than *Pak1^{f/f}* myocytes under chronic β -adrenergic stress ($5.09 \pm 0.26\text{s}$, $n=17$, $p<0.05$) and *Pak1^{cko}* myocytes under baseline conditions ($5.00 \pm 0.21\text{s}$, $n=13$, $p<0.01$) (Fig.3Bi).
2. SR Ca^{2+} content measured by integration of I_{NCX} was reduced in *Pak1^{cko}* than *Pak1^{f/f}* under both baseline and chronic β -adrenergic stress conditions (Fig.3Aii, Bii).
3. Decay measurements from caffeine-induced Ca^{2+} transients (Fig.3Aiii) provided estimates of rates of Ca^{2+} removal by NCX from the cytosol, k_{NCX} (Fig.3Biii). k_{NCX} in *Pak1^{cko}* ($0.026 \pm 0.003/\text{s}$, $n=7$) was significantly reduced compared to that in *Pak1^{f/f}* myocytes ($0.033 \pm 0.002/\text{s}$, $n=16$) under baseline conditions. k_{NCX} was

reduced in *Pak1^{f/f}* myocytes ($0.024 \pm 0.003/s$, $n=10$) under chronic β -adrenergic stress compared to baseline conditions. Finally there was no significant difference in k_{NCX} between *Pak1^{f/f}* and *Pak1^{cko}* myocytes under chronic β -adrenergic stress.

4. Decays in systolic Ca^{2+} transients in myocytes stimulated at 0.5Hz (Fig.3Aiv) reflecting kinetics of cytosolic Ca^{2+} removal through both SERCA and NCX ($k_{SERCA+NCX}$) permitted determination of k_{SERCA} as $(k_{SERCA+NCX} - k_{NCX})$. k_{SERCA} in *Pak1^{cko}* myocytes was significantly lower than that of *Pak1^{f/f}* myocytes under both baseline and chronic β -adrenergic stress conditions (Fig.3Biv). k_{SERCA} was reduced by chronic β -adrenergic stress in both *Pak1^{f/f}* and *Pak1^{cko}* but more severely in *Pak1^{cko}* myocytes (Fig.3Biv).
5. Peak systolic $[Ca^{2+}]_i$ (Fig.3Bv) was estimated from the differences between peaks and baselines of systolic Ca^{2+} transients (Fig. 3Aiv) obtained during regular stimulations. This was indistinguishable between *Pak1^{f/f}* and *Pak1^{cko}* under baseline conditions. It was significantly reduced by chronic β -adrenergic stress in both *Pak1^{f/f}* and *Pak1^{cko}*, but more severely so in *Pak1^{cko}* (Fig.3Bv).
6. Diastolic $[Ca^{2+}]_o$ (Fig.3Av) was estimated by comparing baseline cell Ca^{2+} fluorescence signals with that obtained from control regions of interest where cells were absent. It was higher in *Pak1^{cko}* than *Pak1^{f/f}* myocytes under both baseline and chronic β -adrenergic stress conditions. Chronic β -adrenergic stress increased diastolic Ca^{2+} in *Pak1^{cko}* but not *Pak1^{f/f}* myocytes (resting $[Ca^{2+}]_i$ expressed as a % of total cellular $[Ca^{2+}]_i$).
7. The amplitude of contraction (measured using the Ionoptix system to detect sarcomere length, and expressed as % resting sarcomere length) was indistinguishable between *Pak1^{f/f}* ($9.60 \pm 1.13\%$, $n=13$) and *Pak1^{cko}* ($9.77 \pm 0.98\%$, $n=7$) under baseline conditions (Fig.3Ci, Di), but was significantly reduced in *Pak1^{cko}* ($7.11 \pm 0.57\%$, $n=12$, $p<0.05$) compared to *Pak1^{f/f}* ($9.40 \pm 0.82\%$, $n=9$) myocytes under chronic β -adrenergic stress conditions (Fig.3Ci, Di). However, the rate of relaxation of the myocyte expressed as the maximal velocity of sarcomere relengthening ($-dL/dt$), was significantly slower in *Pak1^{cko}* (baseline: $0.27 \pm 0.03 \mu m/s$, $n=7$, $p<0.05$; chronic β -adrenergic stress: $0.33 \pm 0.02 \mu m/s$, $n=12$, $p<0.005$) compared to *Pak1^{f/f}* (baseline: $0.18 \pm 0.02 \mu m/s$, $n=13$; chronic β -adrenergic stress: $0.24 \pm 0.02 \mu m/s$, $n=9$) under both baseline and chronic β -adrenergic stress conditions (Fig.3Ci, Dii).

Together these findings demonstrate that Pak1 deficiency alters cellular Ca^{2+} homeostasis and contractility under both baseline and chronic β -adrenergic conditions, but more significantly in chronic β -adrenergic stress conditions.

8. In contrast, L-type Ca^{2+} channel currents (I_{LTCC}) were indistinguishable between *Pak1^{cko}* and *Pak1^{f/f}* under either baseline or chronic β -adrenergic conditions, but chronic β -adrenergic stress reduced I_{LTCC} in both *Pak1^{cko}* and *Pak1^{f/f}* myocytes (Fig.3Cii, 3Diii).

Together these findings demonstrate that Pak1 deficiency alters cellular Ca^{2+} homeostasis and contractility under both baseline and chronic β -adrenergic stress conditions, but more significant in chronic β -adrenergic stress conditions.

Pak1 deficiency acts through modifying SERCA2 activity

Further results implicated reductions in SERCA2a activity in these alterations in cellular Ca^{2+} homeostasis, particularly the reduced SR Ca^{2+} content and elevated resting $[\text{Ca}^{2+}]_i$. Myocytes were first stimulated at 0.5 Hz over at least 20 pulses to achieve sequences of Ca^{2+} transients with similar amplitudes. 10 mM caffeine was then added to deplete SR Ca^{2+} and stimulation stopped. The caffeine was withdrawn after 45s and stimulation resumed. Recovery of evoked Ca^{2+} signals (Fig.4A-D) over time was then followed to assess SR Ca^{2+} store recovery from its previous depletion by caffeine. *Pak1^{cko}* myocytes (Fig.4B, D) showed more prolonged recovery time courses compared to corresponding *Pak1^{f/f}* (Fig.4A, C), whether under baseline (Fig.4A, B) or chronic β -adrenergic stress conditions (Fig.4C, D). Chronic β -adrenergic stress accentuated these differences as well as increasing recovery times in both *Pak1^{cko}* and *Pak1^{f/f}* (*Pak1^{f/f}*, baseline vs. chronic β -adrenergic stress, $p = 0.001$; *Pak1^{cko}*: baseline vs. chronic β -adrenergic stress, $p = 0.001$). In contrast, *Pak1^{f/f}* and *Pak1^{cko}* myocytes showed indistinguishable I_{LTCC} and I_{NCX} magnitudes and time courses whether from hearts under baseline or chronic β -adrenergic stress conditions (Fig.4A-D(iii, iv)), and whether at the outset, the middle or following full recovery from caffeine treatment. The bar graphs (Fig.4E-F) represent recovery time courses of the Ca^{2+} transient and the amount of Ca^{2+} in SR to reach steady state.

Pak1 deficiency reduces SERCA2 expression at the transcriptional level

Quantitative PCR and Western blot determinations of mRNA and protein levels of SERCA2a and other key Ca^{2+} handling proteins in *Pak1^{f/f}* and *Pak1^{cko}* hearts under both baseline and chronic β -adrenergic stress conditions (Fig.5Ai) attributed this reduced SERCA2a activity to alterations in both SERCA2a mRNA and and SERCA2a protein levels in *Pak1^{cko}* during chronic β -adrenergic stress. Thus, SERCA2a protein (Fig.5Aii) and mRNA (*atp2a2*) expression (Fig.5Bi) were significantly down-regulated in *Pak1^{cko}* but not *Pak1^{f/f}* hearts under chronic β -adrenergic stress (67% and 59% decrease respectively) consistent with a regulation by Pak1 at the transcriptional level (Fig.5B). Furthermore, total PLN protein levels and its phosphorylation status (at site serine 16) (Fig.5Aiii, iv) and mRNA levels of *pln* (Fig.5Bii) were indistinguishable between *Pak1^{f/f}* and *Pak1^{cko}* hearts whether under either baseline or chronic β -adrenergic stress conditions, making altered PLN action on SERCA2a activity unlikely. Furthermore, in contrast to *Pak1^{f/f}* hearts, *Pak1^{cko}* hearts failed to up regulate either NCX protein (Fig.5Av) or NCX mRNA (*slc8a1*) expression (Fig.5Biii) in response to hypertrophic stress. Finally, there were no differences between *Pak1^{f/f}* and *Pak1^{cko}* whether under baseline or chronic β -adrenergic stress conditions in Nav1.5 protein levels (Fig.5Avi) or *Ryr2* (Fig.5Biv) and *Cacna1c* mRNA levels (Fig.5Bv).

Experiments investigating the mechanism by which *Pak1* regulates *SERCA2a* expression demonstrated that introduction of *Ad-shPak1* achieved a ~60% knockdown of Pak not observed in neonatal rat cardiomyocytes (NRCMs) subjected to control, *Ad-shC2*, virus

(Fig.6A). The known Pak1 activator phenylephrine⁶ then increased SERCA2a protein and mRNA (*atp2a2*) levels (Fig.6Bii, iii). However, this effect was absent in *Ad-shPak1* infected NRCMs (Fig.6B, $P < 0.05$). These findings together implicate Pak1 in transcriptional regulation of *SERCA2a*.

These findings also correlated with observed cytoplasm to nuclear translocations of serum response factor (SRF). This transcriptional factor is known to assume a vital role in gene regulation of cardiogenesis^{9, 10}. In the absence of stimuli, SRF was localized in the cytoplasm following either *Ad-shC2* or *Ad-shPak1* infection. Control *Ad-shC2*-infected NRCMs showed SRF translocation into the nucleus with phenylephrine treatment (Fig.7A, $P = 0.01$). *Ad-shPak1*-treated NRCMs showed a reduced SRF cytoplasm to nuclear translocation accompanied by an increased SRF cytoplasmic intensity (Fig.7A). Analysis of nuclear versus cytoplasmic intensity ratios further supported the finding that an absence of Pak1 attenuated the cytoplasm to nuclear translocation of SRF (Fig.7A: a 62% reduction in nuclear/cytoplasmic intensity ratio) as further illustrated in Fig.7B.

Further evidence implicating SRF as the intermediate responsible for *Pak1*-mediated transcriptional regulation of *SERCA2a* was obtained in NRCMs infected with adenovirus over-expressing constitutively active Pak1 (*Ad-CAPak1*) or *Ad-LacZ* following transfection with either control siRNA (siC2) or siSRF for 72 h. *Ad-CAPak1*-infected myocytes exhibited a 38% increase in *SERCA2a* protein levels in contrast to *Ad-LacZ*-infected myocytes (Fig.8A). Moreover, NRCMs treated with the control siC2 followed by *Ad-CAPak1* infection showed increased *SERCA2* protein (by 72%) and mRNA levels (by 85% compared to *Ad-LacZ* treated cells). In contrast, in NRCMs transfected with siSRF for 72h, which achieved approximately 65% knock-down of SRF (Fig.8B), that were exposed to constitutively active *Pak1* through *Ad-CAPak1*, failed to show a *SERCA2a* up-regulation whether at protein or transcriptional levels (Fig.8C).

Discussion

Our whole animal, tissue, cellular and molecular studies together demonstrate for the first time key regulatory roles for *Pak1* in cellular adaptation to acute, and chronic adrenergic stress, through regulatory actions on *SERCA2a* accordingly critical to myocardial Ca^{2+} homeostasis and electrophysiological stability. These likely involve transcriptional mechanisms involving regulation by SRF. Our studies provide translational platforms for designing novel *SERCA2a* based therapeutic strategies for ventricular tachy-arrhythmias associated with myocardial dysfunctional conditions associated with adrenergic stress, cardiac hypertrophy and HF.

Our electrophysiological studies in intact animals and isolated hearts independently implicated *Pak1* in maintaining ventricular electrophysiological stability during both acute, and chronic adrenergic stress through acting on triggered activity rather than re-entry. Concurring with findings in total *Pak1* knockout hearts¹¹, single *Pak1*^{cko} cells showed correspondingly increased diastolic $[\text{Ca}^{2+}]_i$, decreased SR Ca^{2+} content, prolonged SR Ca^{2+} refilling and impaired contractility under both baseline and chronic β -adrenergic stress conditions, and reduced systolic $[\text{Ca}^{2+}]_i$ with chronic β -adrenergic stress, despite normal

LTCC. Our previous study indicates that there are no difference in cardiac hemodynamic features in *Pak1^{cko}* mice compared to *Pak1^{f/f}* mice under baseline conditions¹¹. This implicates functional impairments in SERCA2a, despite the absence of changes in total PLN protein, its phosphorylation status, and its mRNA levels.

Molecular studies then associated *Pak1^{cko}* with downregulated *SERCA2a* transcription during hypertrophic stress. Studies in NRCMs implicated *Pak1* in regulation of SERCA2a expression: *Ad-CAPak1*- but not *Ad-LacZ* infected myocytes showed increased SERCA2a protein. Further data implicated SRF as the intermediate responsible for this regulation: myocytes treated with the control virus siC2, but not with SRF knockdown virus siSRF, showed increases in SERCA2 mRNA and protein following *Ad-CAPak1* infection. Detailed mechanistic links thus merit future exploration: these could involve a previously demonstrated SRF binding to the CArG box within the serum response element SRE^{9, 10}. *Pak1* could either directly activate SRF, or interact with SRF cofactors including Elk1-1 and SAP-1, Elk-1 and SAP-1 thereby enhancing SRF binding to the SRE promoter^{10, 12, 13}. Thus, in murine overexpression of mutant SRF unable to bind SRE reduced SERCA2 mRNA¹⁴; cardiac-specific SRF overexpression led to cardiomyopathy and decreased SERCA2 mRNA expression¹⁵.

These novel *Pak1* effects complement its previously established actions upon the phosphatase PP2A and the resulting balance between kinase and phosphatase activity controlling LTCC and i_K activity. High basal PKA-dependent phosphorylation drives the rhythmic internal Ca^{2+} store oscillations and spontaneous beating in SAN pacemaker cells¹⁶; PP2A modulation exerts anti-adrenergic and anti-hypertrophic effects through MKK4/MKK7/JNK signalling in ventricular cells¹⁷.

Recent studies¹⁸⁻¹⁹ suggest that *SERCA2a* gene therapy may both improve contractile function and restore electrophysiological stability in HF. Our findings accordingly suggest that *Pak1* signaling offers a platform for designing novel SERCA2a-based therapies for ventricular tachyarrhythmias associated with cardiac hypertrophy and HF. Indeed in our preliminary experiments, *Pak1* activation rescued tachyarrhythmic tendency in *ex vivo* electrophysiological studies of *Pak1^{f/f}* and *Pak1^{cko}* mice following maladaptive Ang II induced cardiac hypertrophy. Similarly, the *Pak1* agonist FTY-720, currently FDA-approved for treating multiple sclerosis, was anti-arrhythmic in ischemic rat hearts, preventing or even reversing pre-existing cardiac hypertrophy⁶⁻⁸. This approach would complement current translational developments of *SERCA2a* gene therapy to enhance its expression and/or activity for restoring mechanical, contractile function as opposed to arrhythmia in failing hearts in HF currently under clinical trials.

Taken together, our findings indicate that *Pak1* is required to maintain ventricular Ca^{2+} homeostasis and electrophysiological stability and implicate *Pak1* as a novel regulator of cardiac SERCA2a through a transcriptional mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

In vivo and *ex vivo* electrophysiological studies of *Pak1^{f/f}* and *Pak1^{cko}* hearts under acute or chronic β -adrenergic stress conditions induced by acute or chronic administration of ISO). **A:** Examples of *in vivo* ECG recordings of *Pak1^{f/f}* and *Pak1^{cko}* hearts under baseline conditions. Occasional ectopic beats were observed in *Pak1^{cko}* hearts. **B:** ECG parameters in *Pak1^{f/f}* and *Pak1^{cko}* hearts. *Pak1^{cko}* mice showed significantly increased HR **C:** *In vivo* ECGs of *Pak1^{f/f}* and *Pak1^{cko}* with frequency-dependent pacing under baseline and acute β -adrenergic (ISO) stress conditions. **D:** Summary of the occurrence of VT in *Pak1^{f/f}* and *Pak1^{cko}* with frequency-dependent pacing under baseline and acute β -adrenergic stress conditions. *Pak1^{cko}* hearts showed higher occurrences of VT and acute β -adrenergic stress conditions. *Pak1^{cko}* hearts showed higher occurrences of VT under higher pacing frequencies, particularly under acute β -adrenergic stress. **E:** Examples of MAP recording from *ex vivo* hearts of *Pak1^{f/f}* and *Pak1^{cko}* under baseline and acute β -

adrenergic stress conditions. **Ei-ii:** $Pak1^{cko}$ hearts showed spontaneous VT under acute ISO stress during sinus rhythm,. **Eiii-Eiv:** paced $Pak1^{cko}$ hearts showed VT under acute β -adrenergic stress. **F:** MAP recordings from *ex vivo* hearts of $Pak1^{f/f}$ and $Pak1^{cko}$ under chronic β -adrenergic stress. AP alternans and polymorphic VT under burst pacing shown by $Pak1^{cko}$ hearts (**Fii**) and (**Fiv**). These are absent in $Pak1^{f/f}$ hearts (**Fi** and **Fiii**). **G** and **H:** Summary of occurrence of VT and values of VERP in the *ex vivo* MAP recording of $Pak1^{f/f}$ and $Pak1^{cko}$ hearts under baseline, acute and chronic β -adrenergic stress conditions.

Figure 2.

Ca²⁺ transients in field-stimulated *Pak1^{f/f}* and *Pak1^{cko}* myocytes under baseline and chronic β -adrenergic stress conditions induced by chronic administration of ISO. **A:** Ca²⁺ transients under 0.5 Hz (**Ai**), 1 Hz (**Aii**) and 3 Hz stimulation (**Aiii**). The occurrence of Ca²⁺ waves increases with pacing frequency in both *Pak1^{f/f}* and *Pak1^{cko}* myocytes, but show significantly higher incidences in *Pak1^{cko}* myocytes particularly under chronic β -adrenergic stress. **B:** Occurrence of Ca²⁺ waves expressed as percentages in *Pak1^{f/f}* and *Pak1^{cko}* myocytes under baseline and chronic β -adrenergic stress conditions.

Figure 3.

Effects of Pak1 deficiency on Ca^{2+} homeostasis and contractility. **A:** records of I_{NCX} (**Ai**), SR Ca^{2+} content (**Aii**), caffeine-induced Ca^{2+} transients reflecting I_{NCX} kinetics (**Aiii**) systolic Ca^{2+} transients (stimulated at 0.5 Hz) from $\text{Pak1}^{\text{f/f}}$ and Pak1^{cko} myocytes (**Aiv**), diastolic Ca^{2+} (**Av**) under baseline and chronic β -adrenergic stress conditions induced by chronic administration of ISO. **B:** Time decays of I_{NCX} of Pak1^{cko} and $\text{Pak1}^{\text{f/f}}$ under baseline and β -adrenergic stress conditions (**Bi**), SR content (**Bii**), I_{NCX} kinetics (**Biii**) and SERCA kinetics (**Biv**), systolic Ca^{2+} (**Bv**). **C:** the representative sarcomere shortening traces of Pak1^{cko} and $\text{Pak1}^{\text{f/f}}$ under baseline and chronic β -adrenergic stress conditions (**Ci**) and LTCC currents (**Cii**). **D:** peak shortening amplitude of sarcomere length (measured as peak shortening normalised to resting sarcomere length) (**Di**), the maximal velocity of sarcomere relengthening ($-\text{dL}/\text{dt}$) (**Dii**) and LTCC current density (**Diii**).

Figure 4.

Recovery of SR Ca^{2+} stores following previous depletion by addition of caffeine. Ca^{2+} transients reflecting SR refilling in $\text{Pak1}^{\text{fl/fl}}$ and Pak1^{cko} myocytes under baseline (**Ai** and **Bi**) and chronic β -adrenergic stress condition induced by chronic administration of ISO (**Ci** and **Di**). Myocytes were stimulated at 0.5 Hz until stable Ca^{2+} transients were reached. The SR was emptied by 10 mM caffeine for 45 s, and the stimuli then re-applied. Three points (a, b, c) were chosen for each SR refill stage representing the beginning, mid-way and the end of SR refilling. For each point, the corresponding Ca^{2+} transients (**Aii**, **Bii**, **Cii** and **Dii**), I_{LTCC} (**Aiii**, **Biii**, **Ciii** and **Diii**) and I_{NCX} tail current (**Aiv**, **Biv**, **Civ** and **Div**) are presented below the SR refilling Ca^{2+} transients. Amplitudes of Ca^{2+} transients and I_{NCX} tail current increased gradually from point a to c, whilst I_{LTCC} was obviously altered. Bar graphs

summarise times for SR Ca^{2+} refilling (**E**) and Ca^{2+} transients (**F**) to reach a steady state. *Pak1^{cko}* myocytes take significantly longer times for Ca^{2+} SR and Ca^{2+} transients to recover under both baseline and chronic β -adrenergic stress conditions. The scale bars for **Aii-iv**, **Bii-iv**, **Cii-iv** and **Dii-iv** are presented on the right panels.

Figure 5.

Pak1 increases protein and mRNA expression levels of SERCA2 in response to chronic β -adrenergic stress conditions induced by chronic administration of ISO. **A:** Ventricular extracts from *Pak1^{f/f}* and *Pak1^{cko}* mice were analysed by immunoblotting for SERCA2, NCX1, PLN, phospho-PLN and Nav1.5 (sodium channel) levels. GAPDH expression was used as the protein loading control. Bar graphs represent ratio of each protein against GAPDH (n=4-6 mice per group). **B:** qPCR analyses of mRNA expression levels of *Atp2a2* (SERCA2), *Pln* (PLN), *Slc8a1* (NCX1), *Ryr2* (ryanodine receptor 2) and *Cacna1c* (Cav1.2) normalised to *gapdh* (n=4-6 mice per group, data is presented as mean $\pm$ SEM).

Figure 6.

Activation of Pak1 increases SERCA2 protein and mRNA levels in neonatal rat cardiomyocytes (NRCMs). **A:** Immunoblot analyses of Pak1 protein levels in NRCMs infected with control *Ad-shC2* or *Ad-shPak1* (both at 25 MOI for 24h). GAPDH expression was used as the protein loading control. Bar graphs represent ratio of Pak1/GAPDH. **B:** Immunoblot and qPCR analyses for SERCA2 protein and mRNA expression levels in NRCMs infected with *Ad-shC2* or *Ad-shPak1* (25 MOI) for 16-18h prior to treatment with phenylephrine (PE, 30 μ M) for 48h. Tubulin expression as protein loading control. Bar graphs represent ratio of SERCA2/Tubulin (protein expression) and *atp2a2/gapdh* (mRNA expression) (n=3 independent experiments).

Figure 7.

Activation of Pak1 causes SRF nuclear translocation. **A:** Representative fluorescent images of SRF (red) staining in NRCMs infected with *Ad-shC2* or *Ad-shPak1* (25 MOI for 16-18h prior to PE treatment for 24h) showing SRF nuclear translocation (scale bar 20 μ M); blue for nuclei visualisation. Bar graph show SRF nuclear/cytoplasmic intensity ratio (n=3 independent experiments, 80 cells counted per experiment, data is presented as mean $\pm$ SEM). **B:** Illustration depicting SRF nuclear translocation from the cytosol into the nucleus post exposure to stimuli; thus initiating SERCA2 gene transcription regulation.

Figure 8.

Increased levels of activated Pak1 cannot rescue levels of SERCA2 in the absence of SRF in NRCMs. **A:** Immunoblot analyses of Pak1 and SERCA2 protein levels in NRCMs infected with *Ad-LacZ* or *Ad-CAPak1* (both at 25 MOI for 48h). Tubulin expression as protein loading control. Bar graph represent ratio of SERCA2/Tubulin (n=3 independent experiments). **B:** Immunoblot analyses of SRF protein levels in NRCMs transfected with control siRNA (*siC2*) or SRF siRNA (*siSRF*, 100nM) for 72h (n=3 independent experiments). Tubulin expression was used as the protein loading control. Bar graph represent ratio of SRF/Tubulin. **C:** Immunoblot and qPCR analyses for SERCA2 protein and mRNA expression levels, respectively, in NRCMs transfected with *siC2* or *siSRF* for 48h prior to infection with *Ad-LacZ* or *Ad-CAPak1* (both at 25 MOI) for a further 48h. Tubulin expression was used as the protein loading control. Bar graphs show protein levels of

SERCA2/Tubulin ratio and mRNA levels of *Atp2a2* (SERCA2) normalised to *gapdh*, respectively (n=3 independent experiments).