Epac2 Mediates Cardiac $\beta_1$-Adrenergic Dependent SR Ca$^{2+}$ Leak and Arrhythmia

Running title: Pereira et al.; Epac2 induce $\beta_1$-AR-mediated SR Ca$^{2+}$ leak

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Abstract:

**Background** — β-adrenergic receptor (β-AR) activation can provoke cardiac arrhythmias mediated by cAMP-dependent alterations of Ca$^{2+}$ signaling. However cAMP can activate both PKA and an Exchange protein directly activated by cAMP (Epac) but their functional interaction is unclear. In heart selective Epac activation can induce potentially arrhythmogenic sarcoplasmic reticulum (SR) Ca$^{2+}$ release that involves CaMKII effects on the ryanodine receptor (RyR).

**Methods and Results** — We tested whether physiological β-AR activation causes Epac-mediated SR Ca$^{2+}$ leak and arrhythmias, whether it requires Epac1 vs. Epac2, β$_1$-AR vs. β$_2$-AR and CaMKIIδ-dependent phosphorylation of RyR2-S2814. We used knockout mice for Epac1, Epac2 or both (DKO). All knockouts exhibited unaltered basal cardiac function, Ca$^{2+}$ handling and hypertrophy in response to pressure overload. However, SR Ca$^{2+}$ leak induced by the specific Epac activator 8-CPT in wild-type was abolished in Epac2-KO and DKO, but unaltered in Epac1-KO. β-AR-induced arrhythmias were also less inducible in Epac2-KO vs. wild-type. β-AR activation with PKA inhibition, mimicked 8-CPT effects on SR Ca$^{2+}$ leak, and was prevented by blockade of β$_1$-AR but not β$_2$-AR. CaMKII inhibition (KN93) and genetic ablation of either CaMKIIδ or CaMKII phosphorylation on RyR2-S2814 prevented 8-CPT-induced SR Ca$^{2+}$ leak.

**Conclusions** — β$_1$-AR activates Epac2 to induce SR Ca$^{2+}$ leak via CaMKIIδ-dependent phosphorylation of RyR2-S2814. This pathway contributes to β-AR-induced arrhythmias and reduced cardiac function.

**Key words:** arrhythmias, sarcoplasmic reticulum, heart failure. adrenergic receptors, calcium.
Introduction

β-adrenergic receptor (β-AR) activation (which is chronic in heart failure, HF) can provoke cardiac arrhythmias due to abnormal diastolic Ca$^{2+}$ release from the sarcoplasmic reticulum (SR) via phosphorylation of the ryanodine receptor (RyR2). This also contributes to reduced cardiac contractility caused by a reduced SR Ca$^{2+}$ load and systolic SR Ca$^{2+}$ transient. Early studies of enhanced SR Ca$^{2+}$ leak in HF or with β-AR activation focused on PKA, a classical cAMP target. However, β-AR activation can also induce PKA-independent diastolic SR Ca$^{2+}$ leak mediated by Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII). Indeed, Epac (Exchange protein directly activated by cAMP), a cAMP target, has been shown to increase diastolic SR Ca$^{2+}$ leak via CaMKII. Two Epac isoforms have been identified. Epac1 (one cAMP binding domain) seems ubiquitously expressed whereas Epac2 (two cAMP binding sites) seems more prominent in brain, pituitary and adrenal gland.

To date, cardiac Epac activation, using a cAMP analog 8-CPT (8-(4-chlorophenylthio)-2’-O-methyladenosine-3’,5’-cyclic monophosphate) can enhance SR Ca$^{2+}$ release, via the small-G protein, Rap and phospholipase-Cε. Although most work agrees on CaMKII as the mediator of Epac-dependent RyR2 activation, Epac-induced SR Ca$^{2+}$ leak can also reduce SR Ca$^{2+}$ load and release. Those effects are similar to those observed in HF where Epac is also upregulated. Notably, 8-CPT can trigger ventricular arrhythmias in mice and may be involved in cardiac fibrosis through β-AR-dependent production of IL-6 in cardiac fibroblast. Thus Epac may be intimately involved in cardiomyopathy such as arrhythmias and HF via alteration of Ca$^{2+}$ handling.

Despite the emergence of this cAMP- and Epac-induced SR Ca$^{2+}$ leak pathway, key fundamental issues are still unknown. 1) Most prior work implicating Epac has relied on the
pharmacological selective agonist 8-CPT, which could have off-target effects. 26 2) It is unknown which Epac isoform mediates RyR and arrhythmogenic effects of Epac activation. 3) It is unknown whether these effects are mediated by type 1 or type 2 β-AR (β₁-AR or β₂-AR). 4) It has been proposed that Epac-mediated effects might require higher levels of β-AR activation than for PKA activation. 5) It is unknown whether and to what extent Epac contributes to β-AR induced arrhythmias. 6) It is not known if the Epac-dependent effects on SR Ca²⁺ release require the major cardiac CaMKIIδ isoform or if other isoforms suffice. 7) It is also unclear whether the SR Ca²⁺ release effects of Epac require the putative RYR CaMKII site S2814. Here we generated Epac knock-out (KO) mice and use other genetically altered mice to unequivocally address all of those points.

We demonstrate that Epac-KO did not alter cardiac function, early pressure overload-induced hypertrophy or basal myocyte Ca²⁺ handling. Epac-dependent arrhythmogenic effects were prevented by ablation of Epac2, β₁-AR, CaMKIIδ and RyR2-S2814 phosphorylation. In conclusion, Epac2 mediates β₁-AR-induced cardiac arrhythmias via CaMKIIδ and RyR-S2814 phosphorylation.

Methods

An expanded Method is provided in the Online-only Supplement.

Epac Knockout Mouse Lines

Epac1 and Epac2 KO mice were developed via similar strategies (see Online-only Supplemental Methods). The Epac1-KO mice were also used in a parallel initial project. 27 Epac1/Epac2 double knockout mice (DKO) were obtained by breeding heterozygous Epac1-KO and Epac2-KO mice to generate double null mice. Littermate Epac2 null mice were used as controls. All procedures
were performed in accordance with NIH Guide for the Care and Use of Laboratory Animals and approved by Institutional Animal Care and Use Committee of UCSD.

**CaMKII\(\alpha\)-KO and RyR2-S2814A and S2814D mice**

Homozygous CaMKII\(\alpha\)-KO mice were generated from heterozygous CaMKII\(\alpha\) mice as previously described.\(^\text{28}\) RyR2-S2814A and RyR2-S2824D knock-in mice were previously described\(^\text{29}\) and respectively inhibit or mimic CaMKII phosphorylation at this site on RyR2.

**Myocytes isolation**

Cardiac ventricular myocytes were isolated using the retrograde Langendorff perfusion technique with Type-2 collagenase (Worthington) perfusion. Cells were kept in 1 mM \([\text{Ca}]\) (20-22°C) before starting experiments. Epac-KO cardiomyocytes were isolated from 3 and 10 months old mice. There were no apparent differences between age groups. All procedures were approved by the UC Davis Institutional Animal Care and Use Committee (IACUC).

**Echocardiography**

Anesthetized mice were analyzed by echocardiography and hemodynamics in 8-month old WT and Epac1-KO, 18-month old WT and Epac2-KO mice and 18-month old control and Epac-DKO mice. Hemodynamic analysis was performed in 3-month old Epac1-KO and WT mice and 5-month old Epac-DKO and control mice. Transverse Aortic Constriction (TAC) was performed in either 3-month old male Epac1 or Epac2 and age-matched WT control mice went either through sham operation \((n=3)\) or TAC surgery \((n=6-9)\).

**In Vivo Electrophysiology**

Programmed intracardiac stimulation was performed in age-matched control \((\text{Ctl})\) and homozygous Epac2-KO mice, as described.\(^\text{30}\) The incidence of reproducible sustained VT (episode >10 consecutive beats of VT) was determined \(\pm\)ISO \((\text{ISO}, 0.5\text{mg/kg, i.p.})\).
Line scan confocal microscopy

Ca\(^{2+}\) Transient, SR Ca\(^{2+}\) leak and SR Ca\(^{2+}\) load were assessed in freshly isolated cells loaded with 5 μM Fluo-3AM or Fluo-4AM (Molecular Probes)\(^{12}\) using confocal microscopy (BioRad, Radiance 2100, x40 oil immersion objective). Excitation was at 488nm (Ar Laser) and emission at >505nm. Ad-FRET-based cAMP indicator\(^{31}\) was provided by Dr. Y.K. Xiang (University of California, Davis). Image analysis was performed using ImageJ software and homemade routines in IDL (Interactive Data Language, ITT).

Quantitative PCR

Quantitative PCR was done using GAPDH (QT01658692, Qiagen) as control. Epac1-1 (forward: 5'-CTCTGTCTCTGCCCTGTCC-3'; reverse: 5' CGCAAAGAAAGAGGTTGAGG-3'), Epac1-2 (forward: 5'-TGTTGGTGAGGCTCAATTCTG-3'; reverse: 5'-CCACACCACGCGGCATC-3'), Epac2-1 (forward: 5'-GGCGTACCAGATGACAACCT-3'; reverse: 5'-CCTCCTCAGGAACAAATCCA-3'), Epac2-2(forward: 5'-TGTTAAAGTGTCTGAGACCAGC-3'; reverse: 5'-AAAGGCTGTCCCAATTCCCAG-3')

Antibodies

Epac1 antibody was a gift from Dr Xiaodong Cheng (University of Texas Medical Branch).

Epac2 and GAPDH antibodies were purchased from Santa Cruz.

Statistical Analysis

Data are expressed as mean ± SEM. For multiple group comparisons, a one way Anova test was followed by Bonferroni post-hoc test. A repeated measures Two way Anova test was followed by a Bonferroni post-hoc test to examine the effect of drug application (before vs. after) between multiple groups. Statistical discrimination between two populations (with or without treatment) was evaluated by Student's t-test for independent samples and paired t-tests for matched samples.
Differences were considered significant for p<0.05.

Results

Generation of Epac1 and Epac2-KO Mouse Lines

Epac1 and Epac2-KO mice were generated using the Cre-loxP system by floxing exons 3-5 and 7, respectively (Online-only Supplemental Data Figure 1A,D). Southern blots show correct recombinant ES cell clones for each case (Online-only Supplemental Data Figure 1B,E). Anti-Epac1 antibody confirmed complete deletion of Epac1 in null mouse hearts (Online-only Supplemental Data Figure 1C). However, commercial anti-Epac2 antibodies could not detect homogenate Epac2 protein. Thus Epac2 was first immunoprecipitated from WT and Epac2-KO hearts before blotting. Flag tagged Epac2 protein expressed in 293T cells were used to confirm antibody efficacy. No Epac2 protein was detected in Epac2-KO hearts (Online-only Supplemental Data Figure 1F). Thus, we created Epac1-KO and Epac2-KO mouse lines.

Cardiomyocytes may express Epac1 and Epac2-mRNA at different levels. Quantitative PCR showed higher Epac2-mRNA than Epac1 in both heart homogenates and isolated cardiomyocytes (Online-only Supplemental Data Figure 2A). Epac1 protein level was not altered in Epac2-KO vs. WT control hearts (not shown), nor was Epac2-mRNA altered in Epac1-KO mice (Online-only Supplemental Data Figure 2B), although we could not assess this at the protein level.

Epac is not critical to baseline cardiac function

In vivo baseline cardiac function was normal in all 3 KOs in terms of left ventricular (LV) posterior wall thickness (LVPW), LV internal dimension (LVID) and fractional shortening (FS%), compared to WT for up to 18 months of age (Figure 1, Online-only Supplemental Data...
Table 1). Moreover, heart rates, LV pressure, \(\pm\)dP/dt\textsubscript{max} and response to dobutamine perfusion were unchanged (Online-only Supplemental Data Table 1). None of the deletions appreciably affected basal cardiac function, heart weight to body weight ratio (Figure 1D). Nor was there any significant difference in the transaortic constriction (TAC; 3-4 weeks) induced hypertrophy, cardiac dysfunction (Figure 1E-F, Online-only Supplemental Data Figure 3) or expression levels of SERCA or NCX (in preliminary Western analysis) in either Epac-KO mouse. The lack of differential phenotype discouraged further study of these TAC mice.

At the myocyte level, basal Ca\(^{2+}\) transient amplitude and time constant of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)],) decline (\(\tau\text{twitch}\)) indicative of SR Ca\(^{2+}\)-ATPase function (Figure 2A-B) as well as SR Ca\(^{2+}\) content and Na\(^+\)/Ca\(^{2+}\) exchange function (\(\tau\text{NCX}\); Figure 2A-D) were not significantly different between groups. Diastolic Ca\(^{2+}\) spark frequency (CaSpF, a measure of SR leak) was unaltered among the groups, particularly when normalized to SR Ca\(^{2+}\) load (to account for the intrinsic effect of SR Ca\(^{2+}\) load on CaSpF; Figure 2D-E). So, Epac ablation does not appreciably alter baseline Ca\(^{2+}\) handling. This is consistent with the idea that basal [cAMP] and Epac activation are low in adult ventricular myocytes in the absence of agonists or phosphodiesterase inhibition. It also creates a uniform baseline with which to examine the role of Epac during \(\beta\)-AR activation.

**Epac2, not Epac1, is responsible for PKA-independent SR Ca\(^{2+}\) Leak**

To test which Epac isoform mediates the previously reported 8-CPT-induced increase of SR Ca\(^{2+}\) release,\(^{12}\) we measured CaSpF in isolated ventricular myocytes. Figure 3A shows that when 10 \(\mu\text{M}\) 8-CPT was applied to individual WT myocytes CaSpF was enhanced, as previously described.\(^{12}\) The same was observed in Epac1-KO (especially when normalized to SR Ca load; Figure 3C). In contrast, the 8-CPT-induced increase of CaSpF was totally abolished in Epac2-
KO or DKO mice (Figure 3A,C).

**Epac2 contributes to β-AR-induced cardiac arrhythmias**

Since Epac2 can enhance diastolic SR Ca release we tested whether Epac2 might contribute to β-AR-induced arrhythmias. This was assessed *in vivo* as the ability of programmed electrical stimulation to induce sustained ventricular tachycardia (SVT) before or after isoproterenol (ISO) injection (0.5mg/kg, i.p.). This protocol was intentionally aggressive, such that all WT exhibited SVT (p<0.001 vs. control). This allowed the detection of reduced susceptibility and SVT duration in Epac2-KO vs. WT (50%, p<0.05 vs. WT; Figure 4A-C). Furthermore, Epac2-KO exhibited reduced tendency to VT vs. WT following a burst pacing protocol (Online-only Supplemental Data Figure 4). WT and Epac2-KO mice did not exhibit differences in either heart rate, ECG parameters or refractory periods at baseline or in response to ISO (Online-only Supplemental Data Table 2 and Online-only Supplemental Data Table 3). They was also no difference in ventricular effective refractory period between WT and Epac2-KO mice (±ISO; Online-only Supplemental Data Table 4) suggesting that the arrhythmia is unlikely due to altered repolarization, but may indeed reflect reduced triggered activity in Epac2-KO related to less RyR sensitization.

To complement those *in vivo* arrhythmia measurements, we assessed ISO-induced increases in CaSpF in Epac2-KO cardiomyocytes, reasoning that SR Ca release events may be a key underlying mechanism for these triggered arrhythmias (Figure 4D). ISO increased CaSpF in WT, but this effect was dramatically blunted in Epac2-KO. The residual CaSpF enhancement in Epac2-KO mice was abolished by PKA inhibition using H89 (a potent inhibitor of PKA). ISO-induced Ca$^{2+}$ waves also tended to be less frequent in Epac2-KO vs. WT myocytes (79 ±24% vs. 132 ± 48% increase; NS). In Epac2-KO mice the inhibition of PKA by H89 fully
reversed the effects of ISO on Ca transient amplitude (Online-only Supplemental Data Figure 5) and correction for SR Ca\(^{2+}\) load (Figure 4E) did not alter this interpretation. We conclude that Epac2 activation (in parallel with PKA) contributes significantly to β-AR-induced triggered arrhythmias and the SR Ca release events which may underlie them.

**Epac is activated by moderate levels of β-AR stimulation**

β-AR stimulation activates PKA and Epac, and alters SR Ca\(^{2+}\) leak by phosphorylation of phospholamban (via elevated SR Ca\(^{2+}\) load\(^{32}\)) or of RyR2 (which is controversial).\(^{1,5,32-34}\) To better understand the physiological significance of Epac signaling, we tested direct β-AR stimulation ±PKA inhibition. First, we assessed the isoproterenol concentration ([ISO]) needed for maximal effects on inotropy, lusitropy and Ca\(^{2+}\) sparks (Figure 5A-B). ISO induced a concentration-dependent increase of Ca\(^{2+}\) transient amplitude, CaSpF and \(\tau_{\text{twitch}}\) (indicative of phospholamban phosphorylation effects). The lusitropic effect was maximal at ~100 nM ISO, while Ca\(^{2+}\) transient amplitude and CaSpF were maximal at 100-300 nM ISO. Thus, we focused on the 30-300 nM range of [ISO] with PKA blocked by pretreatment with 2 μM H89. H89 can influence [cAMP] achieved by ISO application because of PKA effects on phosphodiesterases, which was tested in control experiments using a FRET-based cAMP sensor\(^{35}\) (Online-only Supplemental Data Figure 6). H89 slightly increased basal [cAMP], but 30 nM ISO caused a similar cAMP level to be reached in the absence or presence of H89.

H89 pretreatment blocked the ISO-induced increase of Ca\(^{2+}\) transient amplitude, SR Ca\(^{2+}\) load and \(\tau_{\text{twitch}}\) (Online-only Supplemental Data Figure 7), consistent with potent PKA inhibition. In contrast, ISO still greatly increased CaSpF with PKA blocked, at all [ISO] (Figure 5C) and also decreased Ca\(^{2+}\) transient amplitude (Figure 5D). Both of these effects are similar to those observed upon Epac activation by 8-CPT (Figure 5C-D). This implies that most of the
PKA-independent CaSpF enhancement is mediated by Epac. The ISO-mediated decrease in Ca\(^{2+}\) transient amplitude when PKA is blocked is consistent with the idea that PKA is required for the β-AR-dependent positive inotropic effect, but that other β-AR-mediated effects negatively influence Ca\(^{2+}\) transients (e.g. SR Ca\(^{2+}\) unloading via Epac). Notably, 30nM ISO+H89 was sufficient to fully induce the PKA-independent enhancement of CaSpF, comparable to that induced by 8-CPT. This suggests that physiologically relevant β-AR activation may activate the Epac2 pathway in parallel to PKA, and at similar [cAMP], and both pathways contribute to arrhythmogenesis.

**Epac-induced SR leak is activated by β\(_{1}\)-AR**

Next we sought to determine whether β\(_{1}\)-AR or β\(_{2}\)-AR is responsible for mediating the Epac-dependent activation of Ca\(^{2+}\) sparks and arrhythmias\(^{11,12,17}\). **Figure 6** was done in rat myocytes for comparison with mouse data obtained in other figures as well as previous reports. Both 8-CPT and ISO increased CaSpF (Figure 6A) and reduced steady state SR Ca\(^{2+}\) load (Figure 6C) as seen in mouse experiments (Figure 3). The lower SR Ca\(^{2+}\) load in rat myocytes treated with 8-CPT may be secondary to the increase in CaSpF. This would actually feedback to limit the rise in CaSpF (due to lower luminal SR Ca\(^{2+}\)). As in mouse myocytes, in studies with rat myocytes ISO+H89 produce similar effects to that of 8CPT alone (Figure 6A-C). Thus, ISO+H89 is a good treatment to assess β-AR-induced Epac effects independent of PKA.

To determine the β-AR subtype involved, we treated cells with specific β\(_{1}\)- and β\(_{2}\)-AR inhibitors (along with H89) prior to ISO exposure. β\(_{1}\)-AR blockade (CGP-20712A) was able to fully prevent the ISO-induced increase of CaSpF, whereas the response following β\(_{2}\)-AR blockade (ICI-118.551) was not different from ISO+H89 (Figure 6A). Consistent with this result, CGP-20712A also restored Ca\(^{2+}\) transient amplitude (99.4±6.2% of control) vs. 8-CPT or
ISO+H89 (166±23 and 165±22%) (Figure 6B). The 8-CPT or ISO+H89-dependent rise of SR Ca\textsuperscript{2+} leak, results in a decrease in SR Ca\textsuperscript{2+} load in both cases (Figure 6B), which is partially prevented by β\textsubscript{1}-AR block.

**Epac mediates RyR2-S2814 phosphorylation via CaMKIIδ**

Our previous work suggested that the Epac-induced CaSpF enhancement involved CaMKII activation and RyR2 phosphorylation.\textsuperscript{12} However the phosphorylation site and CaMKII isoform required are unknown. RyR2-S2814 has been suggested as an important CaMKII phosphorylation target.\textsuperscript{30,36} To test its requirement for Epac-dependent effects, we used RyR2-S2814 knock-in mice. In those mice S2814 was rendered either non-phosphorylatable (RyR2-S2814A)\textsuperscript{29} or phosphomimetic (RyR2-S2814D).\textsuperscript{30}

In WT mice, CaMKII inhibition (1 μM KN93) blocked the 8-CPT-induced increase of CaSpF and the reduction of SR Ca\textsuperscript{2+} load and Ca transient (Figure 7A-D). The same effects were observed in both knock-in mice, suggesting that this CaMKII target (and presumably the leak induced), is sufficient to depress twitch Ca\textsuperscript{2+} transients. These results prove that phosphorylation of this CaMKII target site is essential for Epac-dependent activation of SR Ca\textsuperscript{2+} leak.

Notably, the S2814A mice behaved most like WT+KN-93, whereas the S2814D mice exhibited much higher basal CaSpF and lower SR Ca\textsuperscript{2+} load (as recently reported\textsuperscript{30}) but were not significantly modulated by 8-CPT. This is consistent with S2814D being phosphomimetic for CaMKII effects at that site.\textsuperscript{37} Moreover, the unaltered baseline Ca\textsuperscript{2+} transient amplitude, despite reduced SR Ca\textsuperscript{2+} load in S2814D mice is consistent with the CaMKII-dependent enhancement of fractional SR Ca\textsuperscript{2+} release at a constant SR Ca\textsuperscript{2+} load and Ca\textsuperscript{2+} current trigger,\textsuperscript{38} and hence allows relatively normal Ca\textsuperscript{2+} transients.

CaMKIIδ is the dominant CaMKII isoforms in cardiomyocytes (70-90%), but other
isoforms (e.g. CaMKIIγ) could phosphorylate certain targets and contribute to the inhibitory effects of KN93. To test whether the Epac2-dependent effect on Ca²⁺ sparks is mediated via CaMKIIδ specifically, we assessed Ca²⁺ transients and sparks in WT vs. CaMKIIδ-KO mice (Figure 7E-F). The 8-CPT-induced increase of CaSpF and decrease of Ca²⁺ transient amplitude were abolished in CaMKIIδ-KO mice. Thus, the Epac-induced SR Ca²⁺ leak effect seems to require CaMKIIδ activity (vs. other isoforms).

Discussion

The relatively specific Epac activator 8-CPT has been shown to induce diastolic SR Ca leak and arrhythmias, and in human HF Epac expression is increased. This makes it important to understand how Epac-dependent arrhythmogenic signaling works. 8-CPT could have off-target effects, but these seem negligible with respect to Ca²⁺ handling because 10 µM 8-CPT had no acute effect on Ca²⁺ transients or Ca sparks in Epac-2 KO and DKO myocytes. We conclude that myocyte Epac2 is essential for the 8-CPT-induced RyR activation, and that Epac1 cannot substitute. The Epac2-mediated effects on RyR can be induced via β₁-AR activation (not by β₂-AR) and contribute significantly (along with PKA) to β-AR activation dependent arrhythmogenesis in hearts and myocytes. The fact that the ISO-induced Ca spark enhancement was partially prevented in Epac2-KO mice and further suppressed by PKA inhibition (Fig 4D) suggests that Epac2 and PKA are the main pathways here. Other modulators like nitrosylation or oxidation do not seem critical (but could become relevant under some conditions).

CaMKIIδ is required for these Epac2-mediated effects, and other CaMKII isoforms (which constitute ~20% of myocyte CaMKII activity) cannot substitute. One specific site on RyR2 (S2814) also seems essential for Epac2-dependent RyR effects, despite the presence of
other phosphorylation sites on RyR2. This provides novel insight into how Epac works in parallel with the classical cAMP/PKA pathway in cardiomyocytes with respect to Ca\textsuperscript{2+} handling and arrhythmias in heart.

**Epac2, a key protein for β-AR-mediated SR Ca leak and arrhythmia**

At baseline, Epac has little impact on cardiac function and Ca\textsuperscript{2+} signaling, because none of the Epac-KO mice exhibited changes in baseline heart weight/body rate ratio, cardiac function or myocyte Ca\textsuperscript{2+} handling (including diastolic SR Ca\textsuperscript{2+} leak, SR Ca\textsuperscript{2+} load or global SR Ca\textsuperscript{2+} release). However, Epac is called into play during acute physiological β-AR activation and this could be exacerbated in pathological contexts, such as chronic β-AR stimulation during HF. Indeed, Epac2-KO mice were less susceptible to triggered arrhythmias upon β-AR activation and their myocytes produced less arrhythmogenic Ca sparks (Figure 4). In contrast, our data here showed that neither Epac-KO significantly altered TAC-induced hypertrophy or the associated cardiac function changes (Fig 1E-F, Online-only Supplemental Data Fig 3A-D). These data are consistent with the idea that Epac activation plays a more significant role in β-AR-induced myocyte Ca\textsuperscript{2+} mishandling and arrhythmias than in the initial stages of hypertrophy. Indeed, Epac activation by 8-CPT in whole hearts increased diastolic SR Ca\textsuperscript{2+} leak and ventricular arrhythmogenesis. We cannot rule out that Epac might be involved in signaling related to HF, and to the extent that this CaMKII/RyR2 pathway might be critical in the transition from compensated hypertrophy to HF as seen in CaMKIIδ-KO mice, functional upregulation of this Epac pathway during cardiomyopathy may contribute to this transition to HF.

We discovered that only Epac2 mediates enhanced SR Ca\textsuperscript{2+} leak and thus might have different signaling pathways than Epac1 in heart. We found greater Epac2 vs. Epac1 mRNA expression in cardiomyocytes, and while Epac1 mRNA is clearly expressed in heart, the
Epac1/Epac2 mRNA ratio decreases in adulthood compared to neonates\textsuperscript{23} making it plausible that Epac2 may have increasing functional effects with age. We anticipate that Epac1 mediates different functions in cardiomyocytes. Indeed, Epac1 is part of a nuclear muscle-specific A-Kinase anchoring protein complex, reinforcing the idea of its role in nuclear signaling.\textsuperscript{42}

**Role of Epac vs. PKA during β-AR-mediated SR Ca leak**

Acute Epac activation with 8-CPT has been reported to either increase\textsuperscript{11, 16} or decrease (present study)\textsuperscript{12, 19} Ca transient amplitude, but this may only be an apparent discrepancy related to experimental protocols. We and others\textsuperscript{12, 19} focused on 8-CPT effects during steady state where Ca transients were decreased. In contrast, larger initial Ca\textsuperscript{2+} transient during the first 20s are also seen,\textsuperscript{11, 16} consistent with RyR activation (that we describe). This is similar to the effect of low concentrations of the RyR sensitizer caffeine,\textsuperscript{43} where initial Ca transients are larger (sensitized release), but those drive greater Ca efflux and progressively lower SR Ca load. If diastolic SR Ca leak is also enhanced, this can result in lower steady state Ca transients than control, as observed with CaMKII activation.\textsuperscript{44, 45} All agree that Epac activation causes CaMKII-dependent RyR sensitization.

Epac activation increases CaMKII-dependent RyR2 sensitivity to Ca\textsuperscript{2+} which paralleled alteration described during HF. Indeed in HF cardiac contraction is reduced, in part because reduced SR Ca\textsuperscript{2+} content attributed to SR Ca\textsuperscript{2+} leak increase via CaMKII, despite a relative sensitization of the RyR.\textsuperscript{6, 8, 21, 46, 47} Thus, Epac may, along with PKA, be an important a downstream effector of β-AR-cAMP mediated effects in HF.

It is thought that Epac activation requires higher levels of cAMP compared to activation of PKA,\textsuperscript{48-50} which would imply that low levels of β-AR activation might preferentially activate PKA, while higher levels would recruit both PKA and Epac. Our data indicate that 30 nM ISO
was sufficient to induce maximal Epac-dependent RyR2 effects, a lower [ISO] than required for maximal PKA-dependent effects (Figure 5). Thus, during β-AR stimulation in myocytes, Epac activation likely overlaps with PKA. Indeed, both ISO-induced arrhythmias and SR Ca\(^{2+}\) leak effects were strongly attenuated in Epac2-KO mice, reinforcing the idea that Epac and PKA are co-activated in β-AR-induced SR Ca leak and arrhythmias. Global β-AR activation (PKA+Epac) enhances SR Ca\(^{2+}\) leak, Ca\(^{2+}\) transients and SR Ca\(^{2+}\) load. In contrast under Epac activation alone, Ca\(^{2+}\) transient and SR Ca\(^{2+}\) load decrease because of higher SR Ca\(^{2+}\) leak. The enhanced SR Ca\(^{2+}\) load and Ca\(^{2+}\) transients with global β-AR activation can be explained by strong PKA effects on Ca\(^{2+}\) current and phospholamban which increase SR Ca\(^{2+}\)-ATPase activity and SR Ca\(^{2+}\) load. Epac-dependent effects may limit the rise of SR Ca\(^{2+}\) load driven by PKA-dependent effects. Consequently, we expected that β-AR-induced inotropy might be slightly enhanced in Epac-KO mice. This effect was not detected (Online-only Supplemental Data Table 1) possibly because the very strong positive inotropic effects of PKA overcome the modest Epac-mediated negative inotropy. Indeed, the Epac-induced RyR sensitization coupled with increased SR Ca\(^{2+}\) load and Ca\(^{2+}\) current could ablate the moderate negative inotropic effect of Epac.

**Epac2 signaling pathway in β-AR-mediated SR Ca leak**

In cardiomyocytes, β₁-AR and β₂-AR coexist. Here, only β₁-AR inhibition blocked Epac-mediated Ca\(^{2+}\) signaling alterations. This agrees with previous work where β₁-AR favors the formation of a Epac/CaMKII/β-arrestin complex\(^{51}\) and where β₁-AR deletion attenuates CaMKII activation.\(^{52}\) Since Epac also activates CaMKII,\(^{11,12,17}\) these findings reinforce Epac and CaMKII signaling downstream of β₁-ARs (at least at the RyR) and the link between CaMKII-dependent SR Ca\(^{2+}\) leak and arrhythmias.\(^{17,24}\) The genetic block of RyR S2814 phosphorylation, CaMKIIδ-KO and KN93 fully abolished 8-CPT-induced SR Ca\(^{2+}\) leak in WT (Figure 7). These
data extend previous studies\textsuperscript{11,12,17} suggesting that Epac-induced RyR2-S2814 phosphorylation depends on the CaMKII\(\delta\) and not other CaMKII isoforms. However, in RyR2-S2814D mice, we observed a larger SR Ca\(^{2+}\) leak at baseline compared to 8-CPT suggesting that Epac may not achieve the maximum RyR2 activation seen in phosphomimetic RyR2-S2814D mice.

In conclusion, our work indicates that \(\beta_{1}\)-AR activates Epac2-dependent SR Ca\(^{2+}\) leak and arrhythmia via CaMKII\(\delta\)-dependent phosphorylation of RyR2-S2814. These findings give new insights into Epac physiology and pathology. Indeed, Epac seems non-essential for baseline cardiac function, and does not significantly attenuate the acute inotropic response to potent \(\beta\)-AR activation or the early hypertrophic response to pressure overload. However, we demonstrate a specific role for the Epac2 isoform in mediating PKA-independent SR Ca\(^{2+}\) leak via \(\beta_{1}\)-AR causing RyR-S2814 phosphorylation by CaMKII\(\delta\) and SR Ca\(^{2+}\) leak. Further investigations will be needed to understand why both Epac and PKA are activated by \(\beta_{1}\)-AR stimulation and the role of Epac1 (nuclear signaling vs. RyR function or transition to HF).

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\textbf{Conflict of Interest Disclosures:} None.
References:


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1999;85:1009-1019.


Figure Legends:

**Figure 1.** Normal cardiac function in Epac1, Epac2 and DKO assessed by echocardiography. A. 8-months WT (n=6) and Epac1-KO (n=7) mice, 18-months WT (n=7) and Epac2-KO (n=6) mice and 18-months control (n = 7) and Epac-DKO (n=7) mice were measured by echocardiography. Between null mice and controls there was no difference in left ventricular wall thickness (LVPWd), left ventricle internal dimension size (LVIDd) at end of diastole (B), cardiac systolic function (C) or heart weight/Body weight ratio (D; Epac1-KO (n=3), Epac2-KO (n=5) and Epac-DKO (n=3)). E. Cardiac function (fractional shortening, FS) and hypertrophy (left ventricular mass/body weight; LVM/BW) in Epac1-KO (n= 8) and (F) Epac2-KO (n=9) vs. WT measured during 3-4 weeks after TAC.
**Figure 2.** Unaltered basal Ca\(^{2+}\) signaling in Epac-KO mice. A. Normalized Ca\(^{2+}\) transient (left) and SR Ca\(^{2+}\) load (right) traces, recorded in confocal microscopy from WT and Epac-DKO mice. B. Mean Ca\(^{2+}\) transient amplitude (F/F\(_0\), where F\(_0\) is diastolic fluorescence). WT (n=31), Epac1-KO (n=17), Epac2-KO (n=39) and Epac-DKO (n=45). C. Mean Ca\(^{2+}\) spark frequency (CaSpF) as number of sparks per 100 \(\mu\)m s\(^{-1}\). WT (n=25), Epac1-KO (n=14), Epac2-KO (n=39) and Epac-DKO (n=34). D. Mean SR Content assessed by 10 mM caffeine-induced fluorescence change (\(\Delta F/F_0\)). WT (n=25), Epac1-KO (n=15), Epac2-KO (n=29) and Epac-DKO (n=14). E. CaSpF normalized to SR Ca\(^{2+}\) load. Number of animals: 7 WT, 6 Epac2-KO, 4 Epac1-KO and 6 Epac DKO (p=NS).

**Figure 3.** Epac2 is critical for Epac-mediated SR Ca\(^{2+}\) leak. A. Mean CaSpF in intact cardiomyocytes. WT±8-CPT (n=7), Epac1-KO±8-CPT (n=13), Epac2-KO±8-CPT (n=21) and Epac-DKO±8-CP1 (n=20). B. SR Ca\(^{2+}\) load. WT±8-CP1 (n=16), Epac1-KO±8-CP1 (n=13), Epac2-KO (n=13), Epac2-KO+8-CPT (n=21), Epac-DKO (n=12) and Epac-DKO+8-CPT (n=19). C. CaSpF normalized to SR Ca\(^{2+}\) load. **p<0.01, ***p<0.001

**Figure 4.** Epac2-KO deletion reduced ISO-dependent increase of pacing-induced ventricular tachycardia. A. Representative simultaneous surface ECG (lead 2) and intra-cardiac ventricular electrogram after ISO (0.5mg/kg) revealed sustained ventricular tachycardia (SVT) in WT and sinus rhythm in Epac2-KO after S1-S2 extra-stimuli at pacing CL of 90ms. B. Mean SVT incidence before and after ISO stimulation. C. Mean duration of SVT under same conditions (n=12 WT and 12 Epac2-KO mice for C and D). D. Percent effect of ISO on CaSpF in isolated cardiomyocytes from WT (n=7) and Epac2-KO±H89 (n=5 and n=12). E. SR Ca load before and
after ISO in same cells than D. *P<0.05, ***P<0.01.

**Figure 5.** Low β-AR PKA-independent stimulation activates Epac-induced SR Ca\(^{2+}\) leak in rat cardiomyocytes. **A.** Average Ca\(^{2+}\) transient amplitude and Ca\(^{2+}\) transient decay time constant (τ) of SR Ca\(^{2+}\) uptake in intact cardiomyocytes without (n=35) or with 30nM (n=9), 100nM (n=10), 300nM (n=8) or 1μM (n=12) ISO. **B.** ISO concentration-response curve of CaSpF for conditions obtained as in A. **C.** CaSpF ±8-CPT and after physiological activation of Epac (ISO+2μM H89). Control (n=8), 8-CPT (n=8), 30nM ISO+H89 (n=4), 100nM ISO+H89 (n=6) and 300nM ISO+H89 (n=6)). **D.** Average Ca\(^{2+}\) transient amplitude in same conditions. *p<0.05.

**Figure 6.** β1-AR is the upstream activator of Epac-induced SR Ca\(^{2+}\) leak. **A.** CaSpF average under 8-CPT (white, n=7), 100nM ISO (n=5), 100nM ISO+2mM H89 (n=7), ISO+H89+300nM CGP-20712A (β\(_2\)-AR block; n=8) and ISO+H89+50nM ICI-118,551 (β\(_1\)-AR block; n=7). Data are percent with respect to control. **B.** Average Ca\(^{2+}\) transient amplitude under 8-CPT (n=10), 100nM ISO (n=8), ISO+H89 (n=9), ISO+H89+CGP-20712A (n=11) and ISO+H89+ICI-118,551 (n=5). **C.** Mean of percent effect of SR Ca\(^{2+}\) content vs. control. *p<0.05, **p<0.01.

**Figure 7.** Nonphosphorylatable RyR2-S2814 and CaMKIIδ deletion abolish Epac-induced SR Ca\(^{2+}\) leak. **A.** CaSpF average from WT (n=16), S2814D (n=9) and S2814A mice (n=10) before and after 8-CPT and ±KN93 (n=9) for WT. **B.** Mean SR Ca\(^{2+}\) content before 8-CPT in WT (n=18), WT-KN93 (n=9), S2814D (n=15) and S2814A mice (n=11) and +8-CPT in WT (n=16), WT-KN93 (n=9), S2814D (n=5) and S2814A mice (n=13). **C.** Ca\(^{2+}\) transient traces (F/F\(_0\)) in WT, S2814A and S2814D. Dotted traces represents same cells with 10mM 8-CPT. **D.** Average
Ca\textsuperscript{2+} transient amplitude for WT (n=20), WT-KN93 (n=9), S2814A mice (n=11) and S2814D (n=13). E. CaSpF average from WT (n=10), CaMKII\delta KO (n=10) before and after 8-CPT. F. Average Ca\textsuperscript{2+} transient amplitude from WT (n=10), CaMKII\delta-KO (n=10) under same conditions. * p<0.05, ** p<0.01.
Figure 1
Figure 2
**Figure 3**

(A) CaSpF (#/s/100μm) levels in different groups: WT, Epac1-KO, Epac2-KO, Epac-DKO, with CTL and 8-CPT treatments. 

(B) SR Ca Load (ΔF/F₀) in the same groups as in (A). 

(C) CaSpF/SR Ca Load ratios in the same groups as in (A).
Figure 4
Figure 5
Figure 6
Figure 7
Epac2 Mediates Cardiac β1-Adrenergic Dependent SR Ca$^{2+}$ Leak and Arrhythmia
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Xander H. T. Wehrens, Ju Chen and Donald M. Bers

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Supplemental Methods

**Generation of Epac Knockout Mouse Lines**

To make an Epac1 targeting vector, two loxP sites were inserted into intron 2 and 5. A 3.8-kb upstream fragment and a 3.8-kb downstream fragment were used as 5' and 3' recombinant arms, respectively. A Neomycin cassette flanked by two FRT sites was inserted into intron 5 as a positive selection. The DTA cassette as a negative selection was used to assist the rate of positive recombinant events. The linearized targeting DNA was electrophoresed into R1 mouse ES cells at UCSD Transgenic and Gene Targeting Core. After G418 selection, genomic DNA of ES cells was digested with restriction enzyme EcoR V and hybridized with a 470-bp DNA fragment, which was amplified using oligonucleotides: 5'-GAAGCCAGGCAACGAGATT-3' and 5'-AGGCACGAGCTTTACGGTAG-3' from upstream of 5' arm. A 17-kb band is represented for wild-type allele and an 8-kb band for recombinant allele. All microinjected into C57BL/6 blastocysts and transferred onto pseudo-pregnant foster mice. Two correct recombinant ES cell clones were generated from mouse ES Cell genomic DNA and verified by DNA sequencing.

DNA fragments were amplified from mouse ES Cell carrier mice with restriction enzyme Hind III and a 9.2-kb band for targeting allele after hybridizing with a 3.8-kb upstream fragment were used as negative selection was used to assist the rate of positive recombinant events. The linearized targeting DNA was electrophoresed into R1 mouse ES cells at UCSD Transgenic and Gene Targeting Core. After G418 selection, genomic DNA of ES cells was digested with restriction enzyme EcoR V and hybridized with a 470-bp DNA fragment, which was amplified using oligonucleotides: 5'-GAAGCCAGGCAACGAGATT-3' and 5'-AGGCACGAGCTTTACGGTAG-3' from upstream of 5' arm. A 17-kb band is represented for wild-type allele and an 8-kb band for recombinant allele. All DNA fragments were amplified from mouse ES Cell genomic DNA and verified by DNA sequencing.

Two correct recombinant ES cell clones were microinjected into C57BL/6 blastocysts and transferred onto pseudo-pregnant foster mice. Male Chimeras were mated with female black Swiss mice and offspring with genotypic transmission (f<sup>inc</sup>/+) were confirmed by PCR. Neomycin cassette was removed by crossing with Flpe deletion mice. Heterozygous Epac1 (+/-) mice were obtained by further crossing with Protagmine-Cre carrier mice<sup>7</sup>. Wild-type (+/-) and Epac1 null (/-) was generated from heterozygous mice breeding. Listed oligonucleotides are used for genotyping analysis. EPP1: 5'-CTGGGCTCTCTCAGATTTGG-3' and EPP2: 5'-CCTCGCTTGTTGGAAGTCTGTA-3' are used to amplify the wild-type band. Frtneo: 5'-AATGGGCTGACCGCTTCCTCGT-3' and EPP4: 5'-GCCATAGCTCAACAACTGC-3' are used for knockout band.

The same strategy was used to make an Epac2 targeting vector. Exon 7 was flanked by two loxP sites. A 3.6-kb upstream and 2.7-kb downstream DNA fragments were used as 5' and 3' arms. Restriction enzyme Hind III was used to digest ES cells genomic DNA to generate a 13.4-kb band for wild-type allele and a 9.2-kb band for targeting allele after hybridizing with a DNA probe at the downstream of 3' arm. Same breeding processes used for Epac1 null mice were used to produce Epac2 null mice. The following oligonucleotides are used for Epac2 genotype analysis. EQP3 (5'-CCTCCCTTCTCTCTCCCTTCTCT-3') and EQP4 (5'-CGTCGCTGATTTTAGTATTATA-3') are used to amplifying type band and Frtneo and EQP4 are for Epac2 knockout band.

To study Epac1/Epac2 double knockout mice, heterozygous Epac1 with Epac2 null mice were used for breeding to generate double null mice. Littermate Epac2 null mice were used as control in whole study. Epac1 KO, Epac2-KO and DKO mice were born at the expected Mendelian ratios, and are viable and fertile. All procedures were performed in accordance with NIH Guide for the Care and Use of Laboratory Animals and approved by Institutional Animal Care and Use Committee of UCSD.

**Myocytes isolation**

Cardiac ventricular myocytes were isolated using the retrograde Langendorff perfusion technique. Animals were heparin-coagulated with intraperitoneal injection of heparin (50 units/100g body weight). Mice were anesthetized in a gas chamber with 5% isoflurane (100%-O<sub>2</sub>). After heart excision, the heart was cannulated above the aortic valve and perfused by gravity. First, the heart was washed for 1 min with oxygenated Tyrode solution containing (mM): NaCl 130, NaH2PO4 0.4, NaHCO3 5.8, MgCl2 0.5, KCl 5.4, glucose 22, Hepes 25 and 0.1ug/ml insulin (pH 7.4 with NaOH) with 0.1 mM EGTA. Then, cardiac ventricular myocytes were digested by Type-2 collagenase (Worthington) perfusion. Cells were dispersed mechanically for 2-3 min and filtered and centrifuged manually for 1 min. Cells were then washed with 0.5 mM CaCl<sub>2</sub>, centrifuged and finally kept in 1xCM (Ca<sup++</sup>) solution for an hour at room temperature (20-22°C) before starting experiments. Epac-KO cardiomyocytes were isolated from 3 and 10 months old mice. There were no differences between age groups. All procedures were approved by the UC Davis Institutional Animal Care and Use Committee (IACUC).

**Echocardiography**

**Hemodynamics of Mouse Heart and hypertrophy induction**

Anesthetized mice were analyzed by echocardiography and hemodynamic as previously described<sup>8</sup>. 8-month old WT and Epac1-KO, 18-month old WT and Epac2-KO mice and 18-month old control and Epac-DKO mice were assessed by echocardiography, 3-months Epac1-KO and WT mice and 5-month old Epac-DKO and control mice underwent hemodynamic analysis. Transverse Aortic Constriction (TAC) was performed as previously described (63). Either 3-months male Epac1 or Epac2 and age-matched wild-type control mice went either through sham operation (n = 3) or TAC surgery (n = 6-9). Heart function was assessed by echocardiography before TAC surgery as baseline, at 1-2 weeks and 3-4 weeks after surgery.

**In Vivo Electrophysiology Study**

The programmed intracardiac stimulation was performed in age-matched control (Ctl) and heterozygous knockout mice (Epac2 KO) as previously described<sup>9</sup>. Briefly, a 1.1F octapolar catheter (EPR-800; Millar Instruments, Houston, Tex) was inserted into right ventricle via the right jugular vein. Right ventricular apex was paced at a CL of 90ms and S1-S2 extra-stimuli pacing protocol was applied to determine the ventricular tachycardia (VT) inducibility at baseline and after isoproterenol (ISO, 0.5mg/kg, i.p.) injection when the heart rate reached the highest rate. An episode of more than 10 consecutive beats of VT was defined as sustained VT (SVT). The incidence of reproducible SVT (at least positive twice among three tests) was determined at baseline and after ISO injection. Fisher's exact test was applied to compare the categorical date. Paired t-test was applied to compare the SVT duration and heart rate before and after ISO within one group. T-
test was used to compare the difference of SVT duration between 2 groups.

**Line scan confocal microscopy**
Freshly isolated cells were loaded with 5 µM Fluo-3 acetoxyethyl ester (Fluo-3AM or Fluo-4AM, Molecular Probes, in a mixture of DMSO and pluronic acid 20%) for 20 min at room temperature. To wash out extracellular dye, myocytes were settled and the supernatant replaced by Tyrode’s solution (in mM: 140 NaCl, 4 KCl, 1.1 MgCl₂, 10 HEPES, 10 glucose, 1.8 CaCl₂; pH=7.4, with NaOH). Experiments were started 20 min later to ensure complete dye de-esterification. Experiments were performed on confocal microscopy (BioRad, Radiance 2100, x40 oil immersion objective) using line scan mode (3 ms). Fluo-4AM and Fluo-3AM were excited with an Argon laser (λex=488nm) and emission was collected at wavelengths >505nm. Ca transients were evoked by field stimulation (1 Hz). Spontaneous Ca sparks were obtained in quiescent cells after Ca transient recording. SR Ca load was assessed by rapid application of caffeine (10 mM) after 1min of steady state pacing. All experiments were done in Tyrode’s solution (in mM: 140NaCl, 4KCl, 1.1MgCl₂, 10HEPES, 10 glucose, 1.8CaCl₂; pH7.4 with NaOH). cAMP production was assessed using a FRET-based cAMP indicator generously provided by Dr. Y.K. Xiang (University of California, Davis) and expressed in cardiac myocytes by adenoviral gene transfer. Image analysis was performed using ImageJ software and homemade routines in IDL (Interactive Data Language, ITT).

**Quantitative PCR**
quantitative PCR was done as previously described. Two sets of primers were used for Epac1 and Epac2 to compare the mRNA expression between Epac1 and Epac2 in the mouse hearts or isolated myocytes. The following primers were used. GAPDH (QT01658692, Qiagen) were used as control. Epac1-1 (forward: 5'-CTCTGTCTCTGCCTGCTTCC-3'; reverse: 5'-CGCAAGAAAGAGTTGAGG-3'), Epac1-2 (forward: 5'-TGTGTTGAGGGTCAATTCTG-3'; reverse: 5'-CCACACGGGGCATC-3'), Epac2-1 (forward: 5'-GGCGTACCAGATGACAACCT-3'; reverse: 5'-GCCGTACCAGATGACAACTC-3'), Epac2-2(forward: 5'-TGTGTTAAGGTGTCCTGAGACCAGCA-3'; reverse: 5'-AAAGGCTGTCCCAATTCCAG-3')

**Antibodies**
Epac1 antibody was a gift from Dr Xiaodong Cheng (University of Texas Medical Branch). Epac2 and GAPDH antibodies were purchased from Santa Cruz.

**Drugs**
Direct activation of Epac was carried out with 1.5 min perfusion of 10 µM8-CPT (Sigma). Physiological activation of Epac was achieved with 30-300 nM isoproterenol (ISO) (Calbiochem) plus PKA inhibitor. Specific PKA inhibition was attained with 30 min pre-incubation with 2 µM H89 (Calbiochem). β-AR discrimination was resolved by using solutions containing ISO, H89 with block of either β₁-AR (300 nM CGP-20712A, Calbiochem) or β₂-AR (50nM ICI-118.551, Tocris). Cells were pre-incubated with β-AR inhibitors for at least 10 min.

**Statistical Analysis**
Data were expressed as mean ± SEM. Statistical discriminations were performed by using paired Student’s t-test, or one ways ANOVA as appropriate. p<0.05 was considered significantly different.
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**Online Table 1.** Hemodynamic results for wild-type and Epac1 null mice, double null and control mice at basal condition or under various concentrations of dobutamine treatments. Values are mean ± SD. None of values show significant difference between WT and Epac1-KO mice and between DKO and control mice (Epac1<sup>+/+</sup>/Epac2<sup>-/-</sup>). HR, heart rate; LVP, maximum end-systolic left ventricle pressure; dP/dt max (mmHg/sec), maximum positive first derivative of LVP; dP/dt min (mmHg/sec), Maximum negative first derivative of LVP. Exp tau, time calculated from isovolumic left ventricle pressure decay.
Supplemental Table 2

**Cardiac electrophysiological parameters**

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<td>QRS (ms)</td>
<td>8.8 ± 0.3</td>
<td>8.8 ± 0.3</td>
<td>8.2 ± 0.3</td>
<td>8.4 ± 0.3</td>
</tr>
<tr>
<td>QTc (ms)</td>
<td>24.4 ± 0.4</td>
<td>22.9 ± 0.7</td>
<td>24.2 ± 0.8</td>
<td>25.2 ± 1.2</td>
</tr>
</tbody>
</table>

n=12 in each group. *P<0.05 vs. Baseline

Supplemental Table 3

**Cardiac electrophysiological parameters**

<table>
<thead>
<tr>
<th></th>
<th>Ctl (n=12)</th>
<th>Epac2-KO (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNRT (ms)</td>
<td>143.8±6.5</td>
<td>147.3±9.5</td>
</tr>
<tr>
<td>AVERP (ms)</td>
<td>46.3±0.8</td>
<td>44.3±1.1</td>
</tr>
</tbody>
</table>

Table III. Sinus node recovery time (SNRT), and the effective refractory period of AV node (AVERP) at baseline.

Supplemental Table 4

**Ventricular effective refractory period**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>ISO</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>23.5±1.6 ms</td>
<td>22.0±1.1 ms</td>
</tr>
<tr>
<td>Epac2-KO</td>
<td>23.3±1.2 ms</td>
<td>21.5±0.9 ms</td>
</tr>
</tbody>
</table>

Table IV. Ventricular effective refractory period comparing (VERP90) between CTL and Epac2-KO mice.
Supplemental Figure 1: Generation of Epac1 and Epac2 knockout mice. A. Targeting strategy for EPAC1. Exons 3-5 (blue rectangle) were flanked by two Loxp sites (triangle). A neomycin cassette (grey rectangle) flanked by two Frt sites (open rectangle) was inserted into intron 5 of the mouse Epac1 gene. A DNA probe (red rectangle) upstream of the 5’ arm was used as a probe for Southern blot analysis. A DTA (diphtheria toxin; black rectangle) cassette was used to assist in correct recombination. B. Detection of wild-type and mutated alleles by Southern blot analysis. DNA from electroporated ES cells was digested with EcoR V and analyzed by Southern blot analysis with the probe diagrammatically represented in A. The 17 kb and 8 kb bands represent wild-type and mutated alleles, respectively. C. Western blot analysis with Epac1 antibody demonstrated complete absence of Epac1 protein in null mouse hearts. D. Similar strategy used to generate Epac2 targeting vector. E. Detection of wild-type and mutated alleles by Southern blot analysis. DNA from electroporated ES cells was digested with HindIII and analyzed by Southern blot analysis with the probe diagrammatically represented in D. The 13 kb and 9 kb bands represent wild-type and mutated alleles, respectively. F. Epac2 protein was immunoprecipitated from WT not from Epac2-KO mouse hearts. Flag tagged Epac2 protein expressed in 293T cells was specifically recognized by anti-Epac2 antibody.

Supplemental Figure 2: A. Expression of Epac1 and Epac2 mRNA in mouse heart and cardiac myocytes. B. Measurements of mRNA were by quantitative real-time PCR using two different sets of primers for Epac1 and Epac2, respectively (see Methods). Changes in Epac2 mRNA expression were measured in WT vs. Epac1-KO mouse hearts and Epac1 was measured in WT vs. Epac2-KO hearts. There was no significant compensatory change in the remaining Epac isoforms in either case. Values are means ± SD of mRNA levels, expressed as ratio to GAPDH and normalized to levels in WT.
Supplemental Figure 3: Epac1 or Epac2 deletion does not protect against hypertrophy after 3 weeks TAC. Echocardiographic parameters average shown in WT vs. Epac1-KO (Left Panel), and WT vs. Epac2-KO (Right Panel).
Supplemental Figure 4: Incidence of triggered ventricular tachycardia was reduced in Epac2-KO mice. A. Representative simultaneous surface ECG (lead 1) and intracardiac ventricular electrogram after isoproterenol (0.5mg/kg, i.p) revealed ventricular tachycardia in Ctl mice and sinus rhythm in Epac2-KO mice during pacing train at cycle length of 90ms. B. Bar graph summarizing the reproducible VT incidence before and after ISO stimulation. N=12 in each group.

Supplemental Figure 5: H89 effects on β-AR-dependent stimulation effect on SR Ca Twitch. WT (n=6), and in Epac2-KO (n=5) ±H-89 (n=11).
Supplemental Fig 6. cAMP detection detected by FRET-based cAMP indicator (ICUE3). A. Representative example of the FRET signal decrease and CFP signal increase upon cAMP binding after 30 nM ISO±H-89. B. Mean of data obtained represented as F_{CFP}/F_{YFP} ratio. Control (n=37), 30 nM ISO (n=14), H89 (n=33), and 30 nM ISO+H89 (n=30). * p<0.05, ** p<0.01

Supplemental Figure 7: H89 treatment blocks β-AR -dependent activation of PKA. A. Correlation between [Ca] transient amplitude and SR Ca load under ISO±H89. B. Correlation between τ of Ca decline (in ms) and [ISO] ± PKA inhibition by H-89. In both conditions ISO was used at 30, 100 and 300 nM and H89 at 2 µM. Control (n=8), 30 nM ISO (n=5), 100 nM ISO (n=10), 300 nM ISO (n=4) or 1 µmol/L (n=9), 30 nM ISO+H89 (n=4), 100 nM ISO+H89 (n=6) and 300nM ISO+H89 (n=6).
Supplemental Reference


