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Research Article

Cardiology

PKA phosphorylates multiple molecules involved in calcium (Ca^{2+}) handling in cardiac myocytes and is considered to be the predominant regulator of β -adrenergic receptor-mediated enhancement of cardiac contractility; however, recent identification of exchange protein activated by cAMP (EPAC), which is independently activated by cAMP, has challenged this paradigm. Mice lacking *Epac1* (*Epac1* KO) exhibited decreased cardiac contractility with reduced phospholamban (PLN) phosphorylation at serine-16, the major PKA-mediated phosphorylation site. In *Epac1* KO mice, intracellular Ca^{2+} storage and the magnitude of Ca^{2+} movement were decreased; however, PKA expression remained unchanged, and activation of PKA with isoproterenol improved cardiac contractility. In contrast, direct activation of EPAC in cardiomyocytes led to increased PLN phosphorylation at serine-16, which was dependent on PLC and PKC ϵ . Importantly, *Epac1* deletion protected the heart from various stresses, while *Epac2* deletion was not protective. Compared with WT mice, aortic banding induced a similar degree of cardiac hypertrophy in *Epac1* KO; however, lack of *Epac1* prevented subsequent cardiac dysfunction as a result of decreased cardiac myocyte apoptosis and fibrosis. Similarly, *Epac1* KO animals showed resistance to isoproterenol- and aging-induced cardiomyopathy and attenuation of arrhythmogenic activity. These data support *Epac1* as an important regulator of PKA-independent PLN phosphorylation and indicate that *Epac1* regulates cardiac responsiveness to various stresses.

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Epac1-dependent phospholamban phosphorylation mediates the cardiac response to stresses

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PKA phosphorylates multiple molecules involved in calcium (Ca²⁺) handling in cardiac myocytes and is considered to be the predominant regulator of β -adrenergic receptor-mediated enhancement of cardiac contractility; however, recent identification of exchange protein activated by cAMP (EPAC), which is independently activated by cAMP, has challenged this paradigm. Mice lacking *Epac1* (*Epac1* KO) exhibited decreased cardiac contractility with reduced phospholamban (PLN) phosphorylation at serine-16, the major PKA-mediated phosphorylation site. In *Epac1* KO mice, intracellular Ca²⁺ storage and the magnitude of Ca²⁺ movement were decreased; however, PKA expression remained unchanged, and activation of PKA with isoproterenol improved cardiac contractility. In contrast, direct activation of EPAC in cardiomyocytes led to increased PLN phosphorylation at serine-16, which was dependent on PLC and PKC ϵ . Importantly, *Epac1* deletion protected the heart from various stresses, while *Epac2* deletion was not protective. Compared with WT mice, aortic banding induced a similar degree of cardiac hypertrophy in *Epac1* KO; however, lack of *Epac1* prevented subsequent cardiac dysfunction as a result of decreased cardiac myocyte apoptosis and fibrosis. Similarly, *Epac1* KO animals showed resistance to isoproterenol- and aging-induced cardiomyopathy and attenuation of arrhythmogenic activity. These data support *Epac1* as an important regulator of PKA-independent PLN phosphorylation and indicate that *Epac1* regulates cardiac responsiveness to various stresses.

Introduction

β -Adrenergic receptor (β -AR) signaling is well established as a primary defense mechanism against acute stress or changes in hemodynamic load; however, its role in cardiac pathogenesis, although studied extensively, remains poorly understood (1). β -AR blockade, rather than stimulation, is beneficial in patients with heart failure, and pharmacological inhibition of the entire β -AR signaling pathway at the receptor level is one of the accepted treatments for heart failure (2). Studies in mouse models overexpressing β -AR, Gs α , or PKA support the usefulness of β -AR blockade in heart failure (3–5). Recently, however, we have developed a mouse model in which type 5 adenylyl cyclase (AC), a major cardiac subtype, is disrupted (*Ac5* KO), and we found that *Ac5* KO showed resistance to the development of heart failure and exhibited increased longevity (6–9), indicating that inhibition of cAMP signaling at the level of AC, not β -AR, may also result in cardiac protection.

Systolic contraction follows activation of sarcolemmal voltage-gated L-type calcium (Ca²⁺) channel during an action potential, resulting in Ca²⁺-influx, which activates cardiac ryanodine receptor (RyR2) Ca²⁺ release channels on the sarcoplasmic reticulum (SR) (major intracellular Ca²⁺ store). Diastolic relaxation occurs with cessation of Ca²⁺ release, and Ca²⁺ sequestration by the SR Ca²⁺-transporting adenosine triphosphatase (SERCA2a) and its regulator, phospholamban (PLN), indicate that phosphorylations of PLN and RyR2 have a central role in modulating Ca²⁺ homeostasis and, therefore, cardiac function (10, 11). PLN is a low-molecular weight phosphoprotein in cardiac SR, and dephosphorylated PLN is an inhibitor of SERCA2a-mediated transport of Ca²⁺. Following β -AR activation, and thus production of cAMP by AC, PKA phosphorylates PLN on serine-16 (10) in addition to RyR2 on serine-2808 and serine-2814 (11). PKA-mediated serine-16 phosphorylation (and thus SERCA2a activation) is the major mechanism of the lusitropic and also inotropic (12) effects of β -AR stimulation in regulating cardiac function (13). In failing hearts, in contrast, decreased PLN phosphorylation, and thus a decrease in Ca²⁺ uptake by SERCA2a, is a central feature (14, 15). Indeed, decreased inhibition of SERCA2a by PLN ablation can prevent progression of heart failure (16, 17).

Authorship note: Satoshi Okumura, Takayuki Fujita, Wenqian Cai, and Meihua Jin contributed equally to this work.

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Table 1
Heart size and cardiac function in *Epac1* KO

	WT (n)	<i>Epac1</i> KO (n)
Age, month	4.2 ± 0.1 (32)	4.2 ± 0.2 (21)
BW, g	30 ± 0.7 (21)	29 ± 0.9 (18)
Tibia, mm	17.5 ± 0.1 (21)	17.4 ± 0.1 (18)
LV/tibia (mg/mm)	5.2 ± 0.2 (20)	5.2 ± 0.2 (18)
LV/BW (mg/g)	3.2 ± 0.1 (20)	3.3 ± 0.2 (18)
LVEDD, mm	4.3 ± 0.03 (31)	4.4 ± 0.1 (18)
LVESD, mm	2.9 ± 0.04 (31)	3.3 ± 0.1 (18) ^A
LVEF, %	70 ± 0.8 (31)	60 ± 1.1 (18) ^A
%FS	35 ± 1.1 (31)	26 ± 0.6 (18) ^A
Max dP/dt, mmHg	9091 ± 240 (17)	7246 ± 226 (16) ^A
Min dP/dt, mmHg	-9110 ± 240 (18)	-6201 ± 125 (14) ^A

Data are mean ± SEM. LVEDD, LV end-diastolic diameter. FS, fractional shortening. ^A*P* < 0.01.

Others have found that decreased inhibition of SERCA2a through PLN ablation or hyperphosphorylation of PLN exaggerated heart failure and arrhythmogenic activity (18–22). Thus, it remains controversial whether or not enhancement of Ca²⁺ uptake through PLN ablation or PLN hyperphosphorylation can inhibit the progression of heart failure. Nevertheless, it is well accepted that β-AR-mediated phosphorylation of PLN plays a central role in regulating cardiac function and also in the pathogenesis of cardiac failure.

But PKA is not the only molecule activated by cAMP. Exchange protein activated by cAMP (EPAC) was recently identified as a new target of cAMP signaling that is activated independently of PKA (23, 24). EPAC has 2 isoforms (*Epac1* and *Epac2*), and *Epac1* is predominantly expressed in the heart. EPAC modulates various cellular functions, including migration, proliferation, exocytosis, and apoptosis, via regulation of RAP1 (25). In cardiac myocytes, EPAC activation induced hypertrophy (26). In adult mouse cardiac myocytes, pharmacological EPAC activation with an EPAC-selective but not isoform-selective cAMP analogue was reported to enhance evoked Ca²⁺ transients through the PLCε/PKCε/CaMKII pathway, and activation of CaMKII via EPAC induced store depletion and enhancement of Ca²⁺ sparks through increased phosphorylation of PLN on threonine-17 and RyR2 on serine-2815 by EPAC via phospholipase C (PLC) ε/PKC ε/Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) (27–29). EPAC2 was also reported to be closely associated with CaMKII, and EPAC2 mediated β₁-AR-induced cardiac arrhythmia via CaMKIIδ (a major isoform of cardiac CaMKII) and RyR2 phosphorylation on serine-2815 (30). However, the isoform-specific role of EPAC in cardiac function and pathogenesis remains poorly understood.

Here, we show that EPAC1, in an additive and independent manner with respect to PKA, phosphorylates PLN and RyR2 to regulate cardiac function. Loss of EPAC1 slightly decreased basal cardiac function, but afforded greater protection against various stresses, including arrhythmogenic stress, whereas loss of EPAC2 did not show cardioprotective effects. Accordingly, selective EPAC1 inhibition may prevent hyperphosphorylation of PLN and RyR2, and this may be an alternative strategy to current β-AR blocker therapy for the treatment of established cardiac failure or arrhythmia.

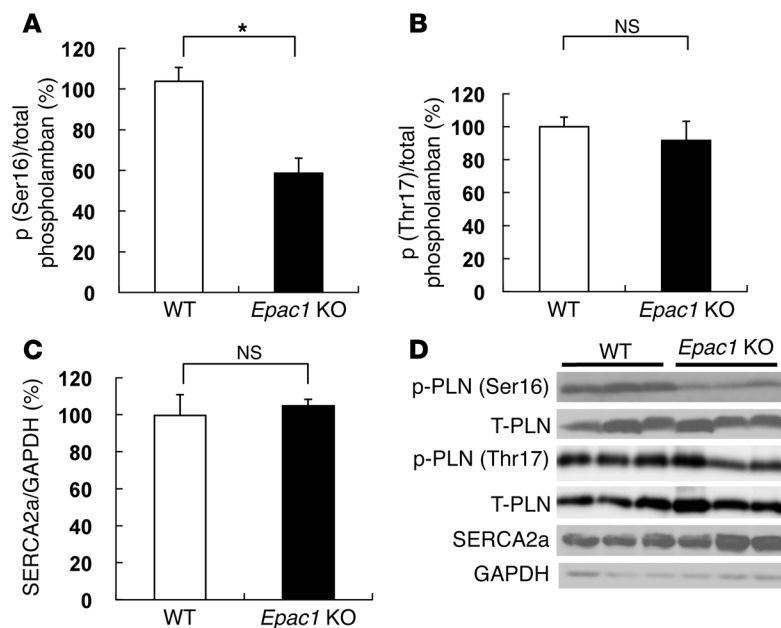
Results

Cardiac function is decreased in Epac1 KO. Because EPAC activation induced cardiac myocyte hypertrophy in culture (26), we originally

thought that EPAC might play a role in regulating cardiac myocyte growth. Therefore, we genetically disrupted *Epac1* in mice (*Epac1* KO) (31), but found that these mice did not show any change in the size of the heart. Instead, *Epac1* KO exhibited a modest, but significant, decrease in cardiac function. LV ejection fraction (LVEF) was significantly decreased (WT versus *Epac1* KO: 70% ± 0.8% versus 60% ± 1.1%, *n* = 18–31, *P* < 0.01), and LV end-systolic diameter (LVESD) was increased (WT versus *Epac1* KO: 4.3 ± 0.03 versus 4.4 ± 0.1 mm, *n* = 18–31, *P* < 0.01) (Table 1). Hemodynamic measurements demonstrated that Max dP/dt was significantly decreased (WT versus *Epac1* KO: 9091 ± 240 versus 7246 ± 226 mmHg, *n* = 16–17, *P* < 0.01), and Min dP/dt was significantly increased in *Epac1* KO (WT versus *Epac1* KO: -9110 ± 240 versus -6201 ± 125 mmHg, *n* = 14–18, *P* < 0.01) (Table 1). Because disruption of *Epac1* might alter the expression of other molecules involved in cAMP signaling, such as PKA subunits, we examined the protein expression of β₁-AR, β₂-AR, β-AR kinase (βARK), Gα, Giα, Gβ, Gγ, and type 5/6 AC in addition to PKA subunits by means of Western blotting (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI64784DS1). There was no difference in expression of these molecules between WT and *Epac1* KO. Also, there was no compensatory increase of *Epac2* isoform in *Epac1* KO at either the mRNA or protein level (Supplemental Figure 1, A and B).

Decreased cardiac function in *Epac1* KO was readily compensated by isoproterenol (ISO) infusion. Responses of LVEF and LVESD to ISO (0, 0.13, 0.27, 0.40 μg/kg/min for 5 minutes) were well preserved and, indeed, reached the same level as those of WT (Supplemental Figure 2, A and B). Changes in cardiac contractility were further examined using isolated segments of myocardium (Supplemental Figure 3). The basal contractile force was significantly smaller in *Epac1* KO (WT versus *Epac1* KO: 112 ± 14.7 versus 73 ± 7.1 mg/mm², *n* = 6–7, *P* < 0.05) (Supplemental Figure 3A). ISO dose dependently enhanced the contractile force in WT and *Epac1* KO. The magnitude of increase in response to ISO was similar in WT and *Epac1* KO (Supplemental Figure 3B). The sensitivity to β-adrenergic stimulation with ISO as represented by the -log EC₅₀ value for the enhancement of contractile force was 7.04 ± 0.07 in WT and 7.06 ± 0.01 in *Epac1* KO (*P* = NS, *n* = 6–7). The time required to reach peak tension was also similar in *Epac1* KO and WT at baseline (WT versus *Epac1* KO: 50 ± 1.1 versus 52 ± 1.5 ms, *n* = 6–7, *P* = NS) and decreased similarly in both WT and *Epac1* KO (Supplemental Figure 3C). However, the time required for 90% relaxation was significantly longer in *Epac1* KO at baseline (WT versus *Epac1* KO: 54 ± 1.5 versus 61 ± 2.0 ms, *n* = 6–7, *P* < 0.05), but became similar in response to ISO (Supplemental Figure 3D). Thus, *Epac1* KO showed decreased cardiac function, exemplified most clearly by delayed relaxation, but the dysfunction was readily compensated by β-AR stimulation.

PLN phosphorylation on serine-16 is decreased in Epac1 KO. Phosphorylation of PLN occurs, following β-adrenergic stimulation, on serine-16 and threonine-17. Indeed, PKA-mediated serine-16 phosphorylation is a key event in increasing cardiac function (32). Unexpectedly, phosphorylation on serine-16 was significantly decreased in *Epac1* KO (WT versus *Epac1* KO: 100% ± 6.5% versus 57% ± 7.1%, *n* = 6, *P* < 0.01) (Figure 1A) despite the absence of any difference in expression of the PKA catalytic and regulatory units (RIα, RIIα) (Supplemental Figure 1A). PLN phosphorylation on threonine-17 (Figure 1B), which is mediated by CaMKII, was similar in WT and *Epac1* KO. Phosphorylation on threonine-286, and thus activation of CaMKII, was also similar (Supplemental Figure 4).

**Figure 1**

Phosphorylation of PLN on serine-16 and threonine-17 and SERCA2a expression in the heart of *Epac1* KO at baseline. (A) Phosphorylation on serine-16 (Ser16) was significantly decreased in *Epac1* KO compared with WT (WT versus *Epac1* KO: 100% ± 6.5% versus 57% ± 7.1%, $n = 6$, * $P < 0.01$). The ratio of phosphorylated/total protein expression of PLN in WT was taken as 100% in each determination. (B) Phosphorylation on threonine-17 (Thr17) was not different in *Epac1* KO and WT (WT versus *Epac1* KO: 100% ± 5.7% versus 92% ± 11.5%, $n = 7-10$, $P = NS$). The ratio of phosphorylated/total protein expression of PLN in WT was taken as 100% in each determination. (C) The expression of SERCA2a protein was not different in WT and *Epac1* KO at baseline (WT versus *Epac1* KO: 100% ± 11.1% versus 105% ± 3.5%, $n = 6-7$, $P = NS$). The ratio of SERCA2a protein/GAPDH in WT was taken as 100% in each determination. (D) Representative immunoblotting results of phosphorylation of PLN on serine-16 (upper) and threonine-17 (middle) and SERCA2a (lower) are shown. T-PLN, total PLN; p-PLN, phosphorylated PLN.

EPAC-selective cAMP analogue increases PLN phosphorylation on serine-16 and threonine-17 in neonatal rat cardiac myocytes. The above findings indicated that disruption of EPAC, but not PKA, decreased PLN phosphorylation. When we examined the consequences of EPAC activation for PLN phosphorylation in neonatal rat cardiac myocytes, we used a new membrane-permeable EPAC-selective but not isoform-selective agonist, i.e., 8-(4-chlorophenylthio)-2'-O-Me-cAMP-AM (8-CPT-AM) (33). We found that 10 μ M 8-CPT-AM was necessary to obtain a significant PLN phosphorylation signal with our detection system in neonatal rat cardiac myocytes (Figure 2D), and thus this concentration was used. This concentration of 8-CPT-AM is in line with those used in previous studies to examine EPAC-mediated signaling, i.e., 10 μ M for adult rat cardiac myocytes (34) or 50 μ M for 293T, JAR, and BeWo cells (35). Furthermore, Brette et al. reported very recently that 8-CPT-AM at 10 μ M did not exert an inhibitory effect on phosphodiesterase (PDE) activity (34). On the other hand, non-AM ester 8-CPT (8-CPT) does inhibit PDE, which is undesirable, since we wish to examine EPAC-specific effects (36, 37).

We found that 8-CPT-AM significantly increased the PLN phosphorylation on serine-16 by approximately 89-fold from baseline and that on threonine-17 by approximately 19-fold from baseline at 15 minutes after the treatment with 8-CPT-AM ($n = 4$, $P < 0.01$) (Figure 2, A and B). PLN phosphorylation on serine-16 was decreased gradually, but was significantly increased even at 120 minutes after 8-CPT-AM treatment. However, PLN phosphorylation on threonine-17 decreased rapidly and did not show a significant increase at 60 minutes after 8-CPT-AM treatment. Thus, activation of EPAC with 8-CPT-AM, in addition to PKA and CaMKII, could induce PLN phosphorylation on serine-16 and threonine-17, but PLN phosphorylation on serine-16 persists for a long time, compared with that on threonine-17, which has been reported to be phosphorylated by EPAC via PLC ϵ /PKC ϵ /CaMKII (27, 28).

Increase of PLN phosphorylation at serine-16 was attenuated in neonatal cardiac myocytes prepared from *Epac1* KO after the treatment of 8-CPT-AM. We examined the effects of 8-CPT-AM on PLN

phosphorylation at serine-16 using neonatal cardiac myocytes prepared from *Epac1* KO and WT. In WT neonatal mouse cardiac myocytes, we found that 1 μ M 8-CPT-AM was sufficient to obtain a significant PLN phosphorylation signal with our detection system (Supplemental Figure 5). Since the magnitude of the increase was similar to that obtained with 10 μ M 8-CPT-AM in neonatal rat cardiac myocytes, we used this concentration (1 μ M) (Figure 2D and Supplemental Figure 5). Epac activation with 8-CPT-AM (1 μ M) significantly increased PLN phosphorylation at serine-16 from baseline in WT, and the level was also significantly greater than that in *Epac1* KO ($P < 0.05$). In contrast with WT, the increase of PLN phosphorylation at serine-16 was not significant (WT: from 100% ± 11% to 1534% ± 528%, $n = 4-6$, $P < 0.01$; *Epac1* KO: from 61% ± 22% to 476% ± 141%, $n = 4-6$, $P = NS$). These results, together with the data shown in Figure 1A and Figure 2A, indicate that EPAC1 is an important regulator of PLN phosphorylation at serine-16, independently of PKA. More importantly, these findings also suggest that 8-CPT-AM at the concentration of 1 μ M phosphorylates PLN at serine-16 via EPAC, not other signaling pathways, such as PKA, cGMP-dependent PKG, or PDE (34, 38).

EPAC phosphorylates PLN at serine-16 via PLC/PKC ϵ . It is established that PKC phosphorylates PLN and that the phosphorylation via PKC is additive to that via PKA and CaMKII, but the significance of these findings and the mechanisms involved remain poorly understood (39-41). It has been suggested that EPAC mediates a novel pathway of regulatory crosstalk between the cAMP and the PLC/PKC pathways (42), and so we hypothesized that EPAC mediates PLN phosphorylation on serine-16 via PLC/PKC. Indeed, in the presence of PLC inhibitor (U73122) or PKC inhibitor (Ro-31-7549), EPAC-mediated phosphorylation of serine-16 was negated in neonatal mouse and rat cardiac myocytes (Supplemental Figure 6). Twelve PKC isoforms are known to exist; conventional PKC subtypes, which include PKC α , PKC β , and PKC γ , require both Ca²⁺ and phospholipid for activation, whereas novel PKC subtypes, which include PKC δ and PKC ϵ , require phospholipid, but not Ca²⁺ (43). We examined the effect of silencing each

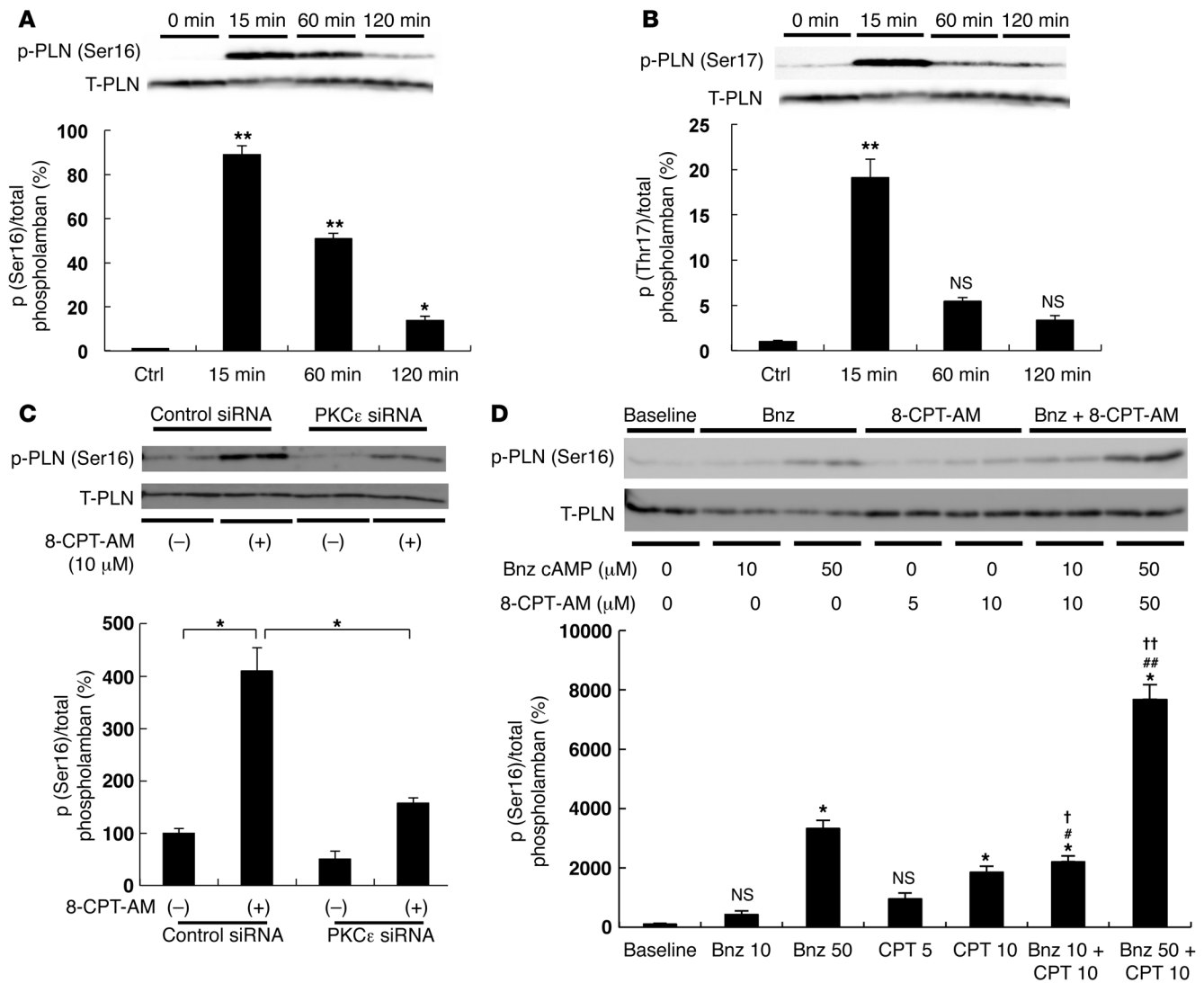
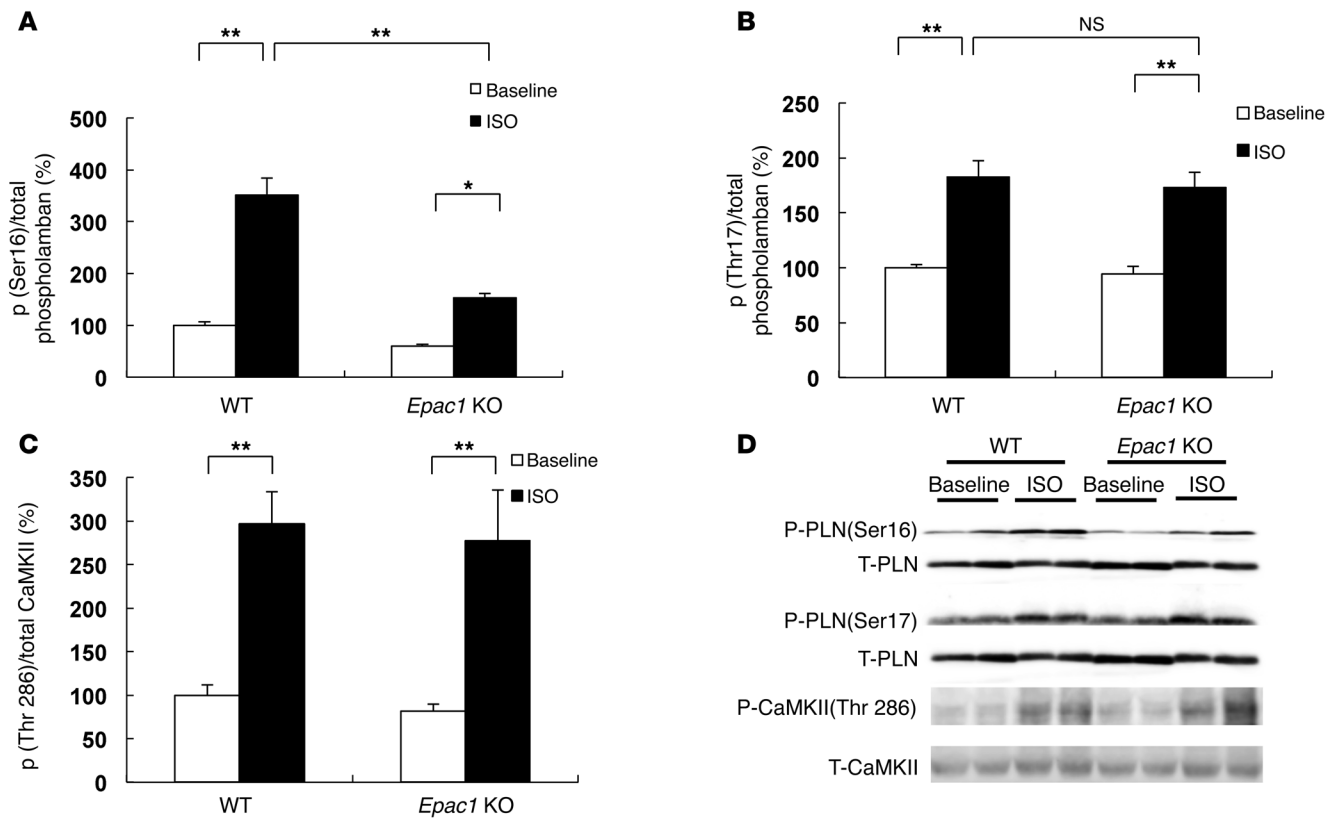


Figure 2

Effects of EPAC activation on PLN phosphorylation in neonatal rat cardiac myocytes. (A and B) Treatment of neonatal rat cardiac myocytes with 8-CPT-AM (10 μM). PLN phosphorylation on serine-16 was significantly increased at 15 minutes and remained significantly ($*P < 0.05$ or $**P < 0.01$ versus 0 minutes, $n = 4$) greater at 120 minutes than at 0 minutes (A). PLN phosphorylation on threonine-17 was also significantly increased at 15 minutes after treatment. However, increase fell below significance at 60 minutes versus 0 minutes and remained unchanged at 120 minutes ($P = NS$, versus 0 minutes, $n = 4$) (B). Ratio of phosphorylated/total protein expression of PLN at baseline (0 min: Ctrl) was taken as 1-fold. (C) EPAC-mediated PLN phosphorylation on serine-16 was examined in neonatal rat cardiac myocytes transfected with PKCε siRNA or control siRNA ($*P < 0.01$, $n = 5-7$). Ratio of phosphorylated/total protein expression of PLN in cells transfected with control siRNA at baseline was taken as 100%. (D) PLN phosphorylation on serine-16 was examined in cells treated with Bnz-cAMP (50 μM) and/or 8-CPT-AM (10 μM). ($##P < 0.001$ versus Bnz-cAMP [50 μM] alone; $††P < 0.001$ versus 8-CPT-AM [10 μM] alone). A similar tendency was observed when 10 μM Bnz-cAMP and 5 μM 8-CPT-AM were used together ($##P < 0.001$ versus Bnz-cAMP [10 μM] alone; $†P < 0.001$ versus 8-CPT-AM [5 μM] alone, $n = 4-8$; $*P < 0.01$ versus baseline, $n = 4-8$). Ratio of phosphorylated/total protein expression of PLN at baseline was taken as 100%.

PKC isoform with siRNA on PLN phosphorylation (Supplemental Figure 7A). Silencing of PKCα, PKCβ, PKCγ, and PKCδ did not affect the EPAC-mediated PLN phosphorylation on serine-16 with 8-CPT-AM (Supplemental Figure 8). However, when PKCε was silenced with siRNA (its specificity was confirmed: Supplemental Figure 7B), EPAC-mediated PLN phosphorylation on serine-16 was significantly attenuated compared with the control (control siRNA versus PKCε siRNA: $410\% \pm 44\%$ versus $158\% \pm 10\%$, $n = 5-7$, $P < 0.01$), suggesting that PKCε plays a role in PLN phosphorylation on serine-16 by EPAC (Figure 2C).

EPAC phosphorylates PLN in an additive and independent manner with respect to PKA. In order to compare the roles of EPAC and PKA in PLN phosphorylation, we examined the effects of N⁶-benzoyladenosine-cAMP (Bnz-cAMP), a PKA-selective cAMP analogue, and 8-CPT-AM, an EPAC-selective analogue (Figure 2D). The degree of PLN phosphorylation achieved with 8-CPT-AM (10 μM) was approximately 60% of that achieved with Bnz-cAMP (50 μM) (Bnz-cAMP versus 8-CPT-AM: $3324\% \pm 289\%$ versus $1851\% \pm 202\%$, $n = 4-8$, $P < 0.001$). However, when 8-CPT-AM and Bnz-cAMP were used together, PLN phosphorylation was additively and significantly increased

**Figure 3**

PLN phosphorylation on serine-16 and threonine-17 and CaMKII phosphorylation on threonine-286 in isolated WT or *Epac1* KO heart perfused according to Langendorf method with or without subsequent ISO (0.1 μ M) for 5 minutes. **(A)** PLN phosphorylation on serine-16 was significantly increased ($*P < 0.05$, $**P < 0.01$) in response to ISO in WT and *Epac1* KO (WT: from 100% \pm 7.0% to 351% \pm 32%, $n = 6-8$; *Epac1* KO: from 60 \pm 2.6 to 153% \pm 9%, $n = 5$), but increase was significantly smaller in *Epac1* KO (153% \pm 9%) compared with WT (351% \pm 32%, $**P < 0.01$, $n = 5-6$). Ratio of phosphorylated/total protein expression of PLN in WT at baseline was taken as 100%. **(B)** PLN phosphorylation on threonine-17 was similar in WT and *Epac1* KO at baseline and was significantly increased in response to ISO in WT (from 100% \pm 2.3% to 183% \pm 14%, $**P < 0.01$, $n = 6-8$) and *Epac1* KO (from 94% \pm 6.7% to 173% \pm 13%, $**P < 0.01$, $n = 6-8$). Magnitudes of the increase were similar ($P = NS$). Ratio of phosphorylated/total protein expression of PLN in WT at baseline was taken as 100%. **(C)** CaMKII phosphorylation on threonine-286 was similar in WT and *Epac1* KO at baseline and was significantly increased ($**P < 0.01$) in response to ISO in WT (from 100% \pm 12% to 297% \pm 37%, $n = 5$) and *Epac1* KO (from 82% \pm 7.7% to 278% \pm 58%, $n = 5$). Magnitudes of increase were similar ($P = NS$). Ratio of phosphorylated/total protein expression of CaMKII in WT at baseline was taken as 100%. **(D)** Representative immunoblotting results of phosphorylation of PLN on serine-16 (upper) and threonine-17 (middle) and CaMKII on threonine-286 (lower). p-CaMKII, phosphorylated CaMKII; T-CaMKII, total CaMKII.

(7688% \pm 497%) relative to that with Bnz-cAMP alone (3324% \pm 289%) or with 8-CPT-AM alone (1851% \pm 202%) ($P < 0.001$ versus Bnz-cAMP or 8-CPT-AM alone). A similar tendency was observed when 5 μ M 8-CPT-AM and 10 μ M Bnz-cAMP were used together, suggesting that EPAC-mediated PLN phosphorylation may be independent of PKA-mediated PLN phosphorylation. Taken together, these results indicate that PLN phosphorylation via EPAC is additive to that via PKA, and both EPAC and PKA might be required for maximal PLN activation via phosphorylation on serine-16.

Effects of infection with Ad-PKI-GFP on PLN phosphorylation in neonatal cardiac myocytes transfected with negative or EPAC1 siRNA. In order to confirm the role of EPAC1 in PKA-independent PLN phosphorylation, we generated recombinant adenovirus of mouse protein kinase inhibitor α -GFP (*Pkia*-GFP) infusion gene (Ad-PKI-GFP) and adenovirus of GFP (Ad-GFP) as controls and examined the effects of Ad-PKI-GFP infection on PLN phosphorylation in neonatal rat cardiac myocytes transfected with control or EPAC1 siRNA (Supplemental Figure 9A, Supplemental Figure 10, and ref. 44).

We first confirmed that more than 98% of cells appeared green and that GFP fluorescence was evenly distributed in the cytoplasm of cardiomyocytes infected with Ad-PKI-GFP or Ad-PKI at MOI 100 (Supplemental Figure 10B).

We next performed Western blotting and found that PLN phosphorylation on serine-16 was significantly increased in cells infected with Ad-GFP or Ad-PKI-GFP in addition to control siRNA transfection after ISO treatment (10 μ M) for 15 minutes. However, the magnitude of the increase was significantly smaller in cells transfected with Ad-PKI-GFP than with Ad-GFP (Ad-GFP [lane 2] versus Ad-PKI-GFP [lane 4]: 60 \pm 2.6 versus 45 \pm 3.1-fold, $P < 0.05$, $n = 4$) (Supplemental Figure 10C). In order to examine the role of EPAC1 in PKA-independent PLN phosphorylation on serine-16, we examined the effect of EPAC1 siRNA transfection and found that silencing EPAC1 significantly decreased the ISO-promoted PLN phosphorylation on serine-16 in cells infected with Ad-PKI-GFP (lane 5: 22 \pm 0.6-fold, $P < 0.01$, $n = 4$). However, pretreatment with KN-93 (10 μ M for 30 minutes), a specific CaMKII inhibitor,

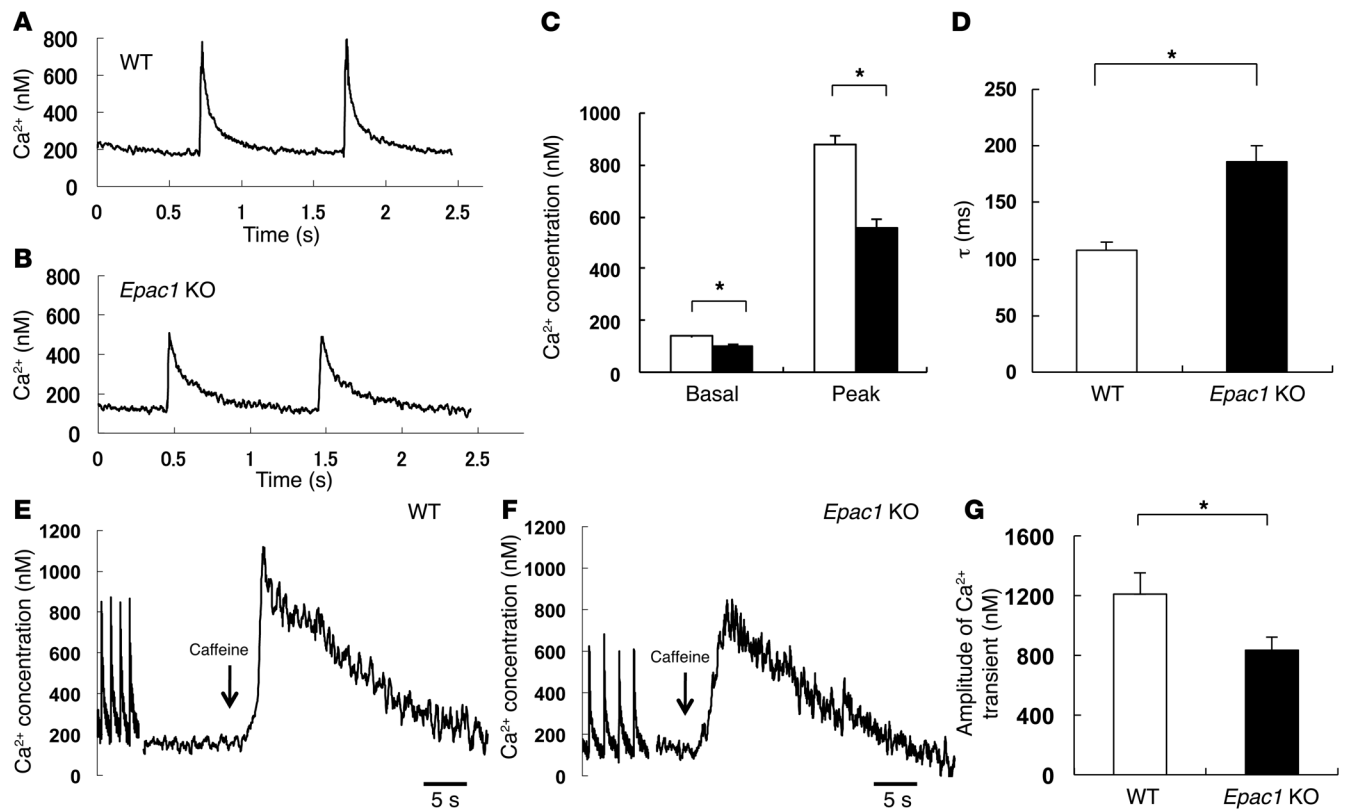


Figure 4

Ca²⁺ transient of adult isolated cardiac myocytes from *Epac1* KO. (A and B) Typical recordings of Ca²⁺ transients in cardiac myocytes from WT (A) and *Epac1* KO (B). Note that the basal and peak Ca²⁺ transient amplitude and decay rate are smaller in *Epac1* KO ($n = 32$ cells from 4 animals each). (C and D) Ca²⁺ transient parameters of isolated cardiac myocytes from *Epac1* KO and WT. The basal Ca²⁺ concentration (WT versus *Epac1* KO: 139 ± 6.8 versus 99 ± 11.8 nM) and peak Ca²⁺ concentration (WT versus *Epac1* KO: 883 ± 35.3 versus 559 ± 33.2 nM) were significantly lower in *Epac1* KO than in WT (C). The decay time constant (τ) was significantly larger in *Epac1* KO than in WT (WT versus *Epac1* KO: 108 ± 6.6 versus 187 ± 13.2 ms) ($n = 32$ cells, * $P < 0.05$) (D). (E and F) Typical recordings of Ca²⁺ transients after caffeine (10 mM) treatment of cardiac myocytes from WT (E) and *Epac1* KO (F). (G) Peak Ca²⁺ concentration after caffeine (10 mM) treatment was significantly decreased in *Epac1* KO compared with WT (WT versus *Epac1* KO: 1210 ± 143 versus 835 ± 87.6 nM, $n = 7$, * $P < 0.05$).

did not decrease ISO-promoted PLN phosphorylation on serine-16 in cells transfected with control siRNA (lane 6: 37 ± 3.1 -fold, $P = \text{NS}$, $n = 8$) (Supplemental Figure 10C).

We also examined whether PLN phosphorylation on threonine-17 was increased in cells transfected with Ad-GFP or Ad-PKI-GFP in addition to control siRNA after ISO treatment (10 μM) for 15 minutes. However, the magnitudes of the increase were similar in both cases (Ad-GFP [lane 2] versus Ad-PKI-GFP [lane 4]: 59 ± 0.04 versus 56 ± 0.05 -fold, $P = \text{NS}$, $n = 8$) (Supplemental Figure 10D). Transfection of EPAC1 siRNA significantly decreased ISO-promoted PLN phosphorylation on threonine-17 in cells transfected with Ad-PKI-GFP (lane 5: 41 ± 0.04 , $P < 0.05$, $n = 8$) as in the case of serine-16. However, KN-93 pretreatment (10 μM for 30 minutes) abrogated the ISO-promoted PLN phosphorylation on serine-17 in cells transfected with control siRNA (lane 6: 1.4 ± 0.003 , $P < 0.01$, $n = 8$) (Supplemental Figure 10D).

Together with the data in Figure 2D, these data obtained with Ad-PKI-GFP clearly demonstrated that EPAC1 and PKA regulate PLN phosphorylation on serine-16 in an additive and an independent manner. Also, EPAC1 regulates PLN phosphorylation at both serine-16 and threonine-17 in vitro, in addition to PKA and CaMKII.

The increase of PLN phosphorylation on serine-16 with ISO was decreased in *Epac1* KO. We next examined the phosphorylation of PLN in total homogenate prepared from WT and *Epac1* KO hearts perfused with the Langendorf method. Hearts were perfused with Krebs solution at constant pressure with or without the treatment of ISO (0.1 μM) for 5 minutes (45), then rapidly frozen in liquid nitrogen for Western blotting (Figure 3).

PLN phosphorylation on serine-16 was significantly decreased in *Epac1* KO compared with that in WT at baseline (WT versus *Epac1* KO: $100\% \pm 7.0\%$ versus $60\% \pm 2.5\%$, $P < 0.05$, $n = 5-8$). It was significantly increased in response to ISO, but the magnitude of the increase was significantly smaller in *Epac1* KO (WT versus *Epac1* KO: $351\% \pm 32\%$ versus $153\% \pm 9\%$, $n = 5-6$, $P < 0.01$) (Figure 3A). PLN phosphorylation on threonine-17 was similar in both WT and *Epac1* KO at baseline ($100\% \pm 3.0\%$ versus $94\% \pm 6.7\%$, $n = 8$, $P = \text{NS}$). However, it was significantly increased in both WT and *Epac1* KO in response to ISO ($P < 0.01$), and the magnitudes of the increase were similar (WT versus *Epac1* KO: $183\% \pm 14\%$ versus $173\% \pm 13\%$, $n = 6$, $P = \text{NS}$) (Figure 3B). CaMKII phosphorylation on threonine-286 was also similar in WT and *Epac1* KO at baseline (WT versus *Epac1*: $100\% \pm 12\%$ versus $82\% \pm 7.7\%$, $n = 5$, $P = \text{NS}$). It was significantly increased in both WT and *Epac1* KO in response

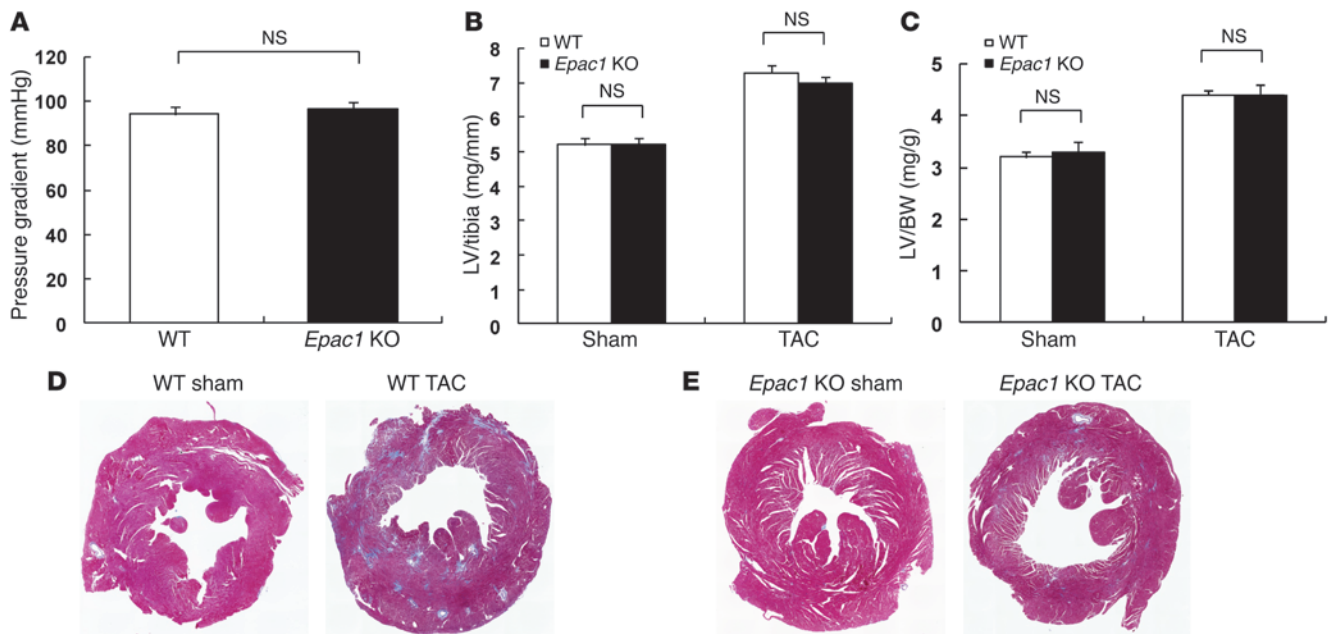


Figure 5

Comparison of cardiac hypertrophy after aortic banding (TAC) in WT and *Epac1* KO. (A) Pressure gradients were not different in WT and *Epac1* KO at 3 weeks after TAC ($n = 13-16$, $P = \text{NS}$). (B and C) LV weight (mg)/tibial length (mm) ratio (B) and LV weight (mg)/body weight (BW, g) ratio (C) were determined at 3 weeks. The degree of cardiac hypertrophy was increased at 3 weeks, but was similar in WT and *Epac1* KO (LV/tibial length ratio for WT versus *Epac1* KO: 7.3 ± 0.2 versus 7.0 ± 0.2 ; LV/BW ratio for WT versus *Epac1* KO: 4.4 ± 0.1 versus 4.4 ± 0.2 , $n = 18-20$, $P = \text{NS}$). (D and E) Representative gross LV section of Masson-trichrome staining in sham-operated and TAC-operated WT and *Epac1* KO heart. Note that the degree of cardiac hypertrophy was similar, but the fibrotic area after aortic banding was smaller in *Epac1* KO than that in WT.

to ISO ($P < 0.01$), and the magnitudes of the increase were similar (WT versus *Epac1* KO: $297\% \pm 37\%$ versus $278\% \pm 58\%$, $n = 5$, $P = \text{NS}$) (Figure 3C). These data indicate that PLN phosphorylation on serine-16, the major site of PKA-mediated phosphorylation, is also regulated by EPAC1. However, EPAC1 does not alter PLN phosphorylation on threonine-17, the major site of CaMKII-mediated phosphorylation, at baseline or in response to ISO.

Silencing EPAC1 in cardiac myocytes attenuates ISO-mediated increases of RyR2 phosphorylations on serine-2808 and serine-2814. PKA and CaMKII-mediated phosphorylation of RyR2 play important roles in modulating cardiac contractility and arrhythmogenesis. It was reported that RyR2 phosphorylation on serine-2808 is mediated by PKA, while RyR2 phosphorylation on serine-2814 is mediated by CaMKII (46). More recently, both sites have been reported to be phosphorylated by PKA and CaMKII, and their phosphorylation is required for adequate RyR2 functional activity (11). We thus examined ISO-induced (10 μM for 3 minutes) phosphorylation on serine-2808 and serine-2814 of RyR2 in neonatal rat cardiac myocytes transfected with control or EPAC1 siRNA because we found that phosphorylated and total RyR2 antibodies did not work well in cardiac homogenate prepared from mouse heart perfused with the Langendorf method (Supplemental Figure 9A and Supplemental Figure 11). RyR2 phosphorylation levels on serine-2808 and serine-2814 were similar in cells transfected with control or EPAC1 siRNA at baseline (serine-2808: control siRNA versus EPAC1 siRNA: $100\% \pm 1.9\%$ versus $130\% \pm 4.9\%$, $n = 4$, $P = \text{NS}$; serine-2814: control siRNA versus EPAC1 siRNA: $100\% \pm 16\%$ versus $116\% \pm 16\%$, $n = 4-6$, $P = \text{NS}$). They were significantly increased in response to ISO, but the magnitudes of the increase on both serine-2808

(Supplemental Figure 11B) and serine-2814 (Supplemental Figure 11C) were significantly smaller in cells transfected with EPAC1 siRNA than those in cells transfected with control siRNA (serine-2808: control siRNA versus EPAC1 siRNA $268\% \pm 8.3\%$ versus $199\% \pm 13\%$; $P < 0.01$, $n = 6$; serine-2814: control siRNA versus EPAC1 siRNA $220\% \pm 13\%$ versus $170\% \pm 2.3\%$, $P < 0.05$, $n = 4-6$). These data indicate that EPAC1 regulates RyR2 phosphorylation on serine-2808 and serine-2814 in addition to PKA and CaMKII.

*Intracellular Ca^{2+} concentration is decreased in *Epac1* KO myocytes.* Cardiac contraction and relaxation are influenced by the intracellular increase in Ca^{2+} during systole and its decrease during diastole. Sarcoplasmic reticulum Ca^{2+} uptake is regulated via PLN phosphorylation. PLN phosphorylation has recently been concluded to play a prominent role in the regulation of myocardial contraction as well as relaxation based on findings in *Pln* knockout mice (12).

We thus examined the intracellular Ca^{2+} concentration in isolated cardiac myocytes of *Epac1* KO (Figure 4, A and B). The basal and peak Ca^{2+} concentrations were significantly decreased in *Epac1* KO compared with WT (basal Ca^{2+} in WT versus *Epac1* KO 139 ± 6.8 versus 99 ± 11.8 nM, $n = 32$, $P < 0.01$; peak Ca^{2+} in WT versus *Epac1* KO: 883 ± 35.3 versus 559 ± 33.2 nM, $n = 32$, $P < 0.01$) (Figure 4C). The decay time constant (τ) was significantly larger in *Epac1* KO than that in WT controls (WT versus *Epac1* KO: 108 ± 6.6 versus 187 ± 13.2 ms, $n = 32$, $P < 0.01$) (Figure 4D). We also examined the cytoplasmic Ca^{2+} concentration after addition of caffeine (10 mM) as a measure of Ca^{2+} content in SR in isolated cardiac myocytes of WT and *Epac1* KO (Figure 4, E-G). The amplitude of the caffeine-induced increase in cytoplasmic Ca^{2+} concentration was significant in WT compared with *Epac1*

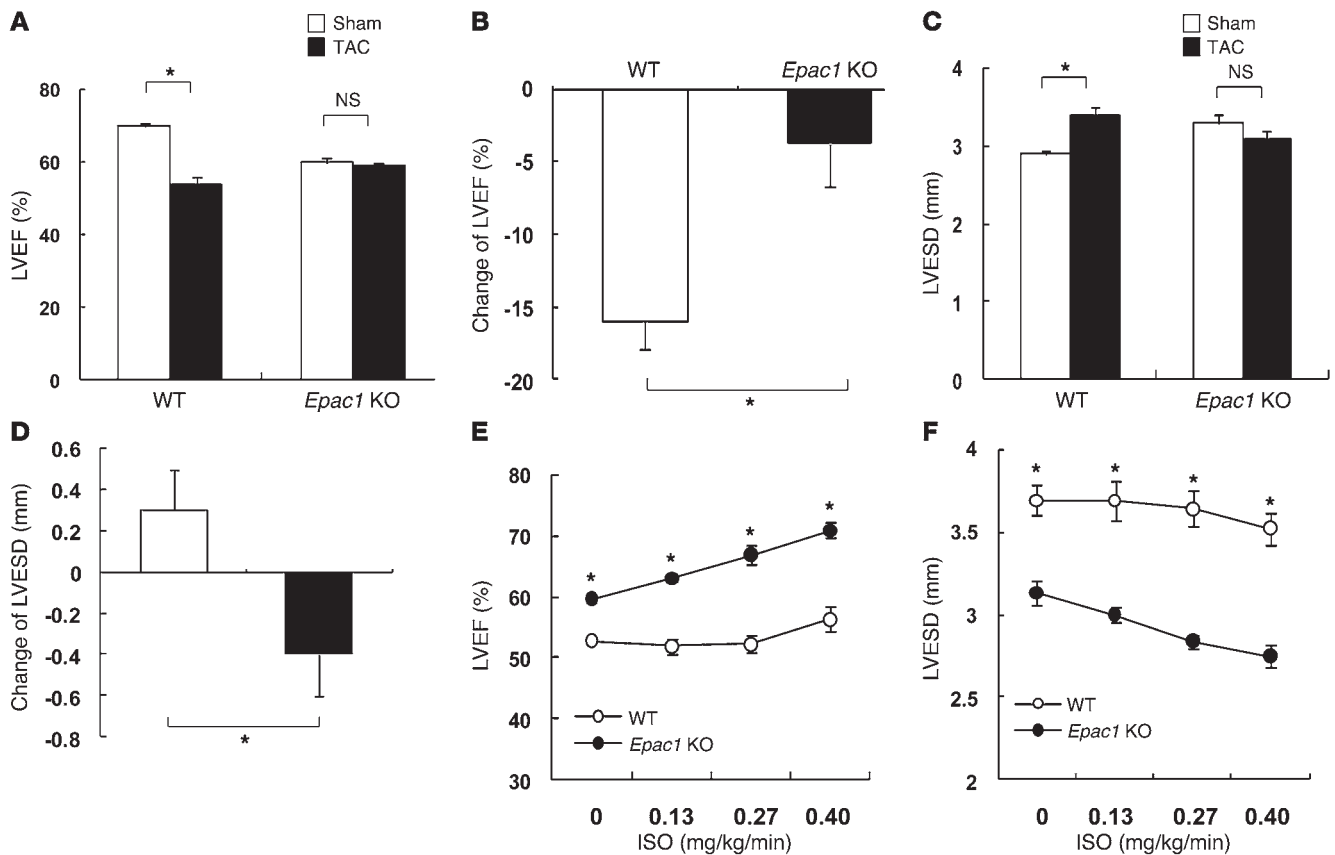


Figure 6

Changes in LV function at 3 weeks after aortic banding (TAC) in WT and *Epac1* KO. Echocardiographic measurements of LV function were performed at 3 weeks after TAC of WT and *Epac1* KO and in sham-operated controls. (A and B) LVEF was significantly decreased in WT (**P* < 0.01), but not in *Epac1* KO (*P* = NS) at 3 weeks (WT versus *Epac1* KO: from 70% ± 0.8% to 54% ± 2.0% versus from 60% ± 1.1% to 59% ± 0.7%, *n* = 17–30) (A). The data were compared with those from sham-operated controls at 3 weeks in each mouse. Change of LVEF from sham-operated controls at 3 weeks after TAC was significantly greater in WT than that in *Epac1* KO (B) (*n* = 17–31, **P* < 0.01). (C and D) LVESD was significantly increased in WT, but not in *Epac1* KO at 3 weeks after TAC (C). The data were compared with those from sham-operated controls at 3 weeks. Change of LVESD from sham-operated controls at 3 weeks after banding was greater in WT than that in *Epac1* KO (*n* = 17–31, **P* < 0.01) (D). The decrease of LVESD in response to intravenous acute ISO infusion (0, 0.13, 0.27, 0.40 mg/kg/min for 5 minutes) was depressed in WT, but not in *Epac1* KO (*n* = 4–5, **P* < 0.01). (E and F) The increase of LVEF (E) and the decrease of LVESD (F) in response to intravenous acute ISO infusion (0, 0.13, 0.27, 0.40 mg/kg/min for 5 minutes) were depressed in WT, but not in *Epac1* KO (*n* = 4–5, **P* < 0.01) (E).

KO (WT versus *Epac1* KO: 1210 ± 143 versus 835 ± 87.6 nM, *n* = 7, *P* < 0.05), indicating that the Ca²⁺ content in SR was decreased in *Epac1* KO compared with WT.

Activities of L-type Ca²⁺ channel and NCX channel in Epac1 KO are similar to those in WT. We measured the L-type Ca²⁺ channel current (*I*_{CaL}) and Na⁺-Ca²⁺ exchange current (*I*_{NCX}) using the cell patch-clamp technique in isolated cardiac myocytes of *Epac1* KO and confirmed that the density and current-voltage relationships of *I*_{CaL} (Supplemental Figure 12) and *I*_{NCX} (Supplemental Figure 13) were not different. We also examined the response of *I*_{CaL}, a PKA-dependent target, in response to ISO (10⁻⁷ M) and confirmed that it was not different between WT and *Epac1* KO (Supplemental Figure 12B and ref. 6).

Disruption of EPAC1 does not affect development of cardiac hypertrophy, but prevents heart failure in pressure overload stress. In order to examine the role of EPAC1 in the development of heart failure, we performed aortic banding in *Epac1* KO and WT for 3 weeks. The pressure gradients were similar in WT and *Epac1* KO at 3 weeks after banding (Figure 5A). At baseline, there was no difference between

WT and *Epac1* KO in the LV weight (mg)/tibial length (mm) (LV/tibial length ratio in WT versus *Epac1* KO: 5.2 ± 0.2 versus 5.2 ± 0.2 mg/mm, *n* = 18–20, *P* = NS). The degree of cardiac hypertrophy was increased at 3 weeks, but remained similar in *Epac1* KO and WT (LV/tibial length ratio in WT versus *Epac1* KO: 7.3 ± 0.2 versus 7.0 ± 0.2, *n* = 18–20, *P* = NS) (Figure 5, B, D, and E). The change of LV/body weight ratio, another index of cardiac hypertrophy, confirmed the finding based on LV/tibial length ratio (WT versus *Epac1* KO: from 3.2 ± 0.1 to 4.4 ± 0.1 versus from 3.3 ± 0.2 to 4.4 ± 0.2 mg/g, *n* = 18–20, *P* = NS) (Figure 5C).

At 3 weeks after banding, however, cardiac function (LVEF) was significantly decreased in WT (*P* < 0.01), while it remained unchanged in *Epac1* KO (WT versus *Epac1* KO: from 70% ± 0.8% to 54% ± 2.0% versus from 60% ± 1.1% to 60% ± 0.7%, *n* = 17–30) (Figure 6, A and B). LVESD was increased significantly in WT at 3 weeks after banding (*P* < 0.01), while it remained unchanged in *Epac1* KO (WT versus *Epac1* KO: from 2.9 ± 0.04 to 3.3 ± 0.1 versus from 3.4 ± 0.1 to 3.1 ± 0.1 mm, *n* = 17–31) (Figure 6, C and D). Hemodynamic measurements demonstrated that Max dP/dt was significantly decreased in WT, but not in *Epac1*

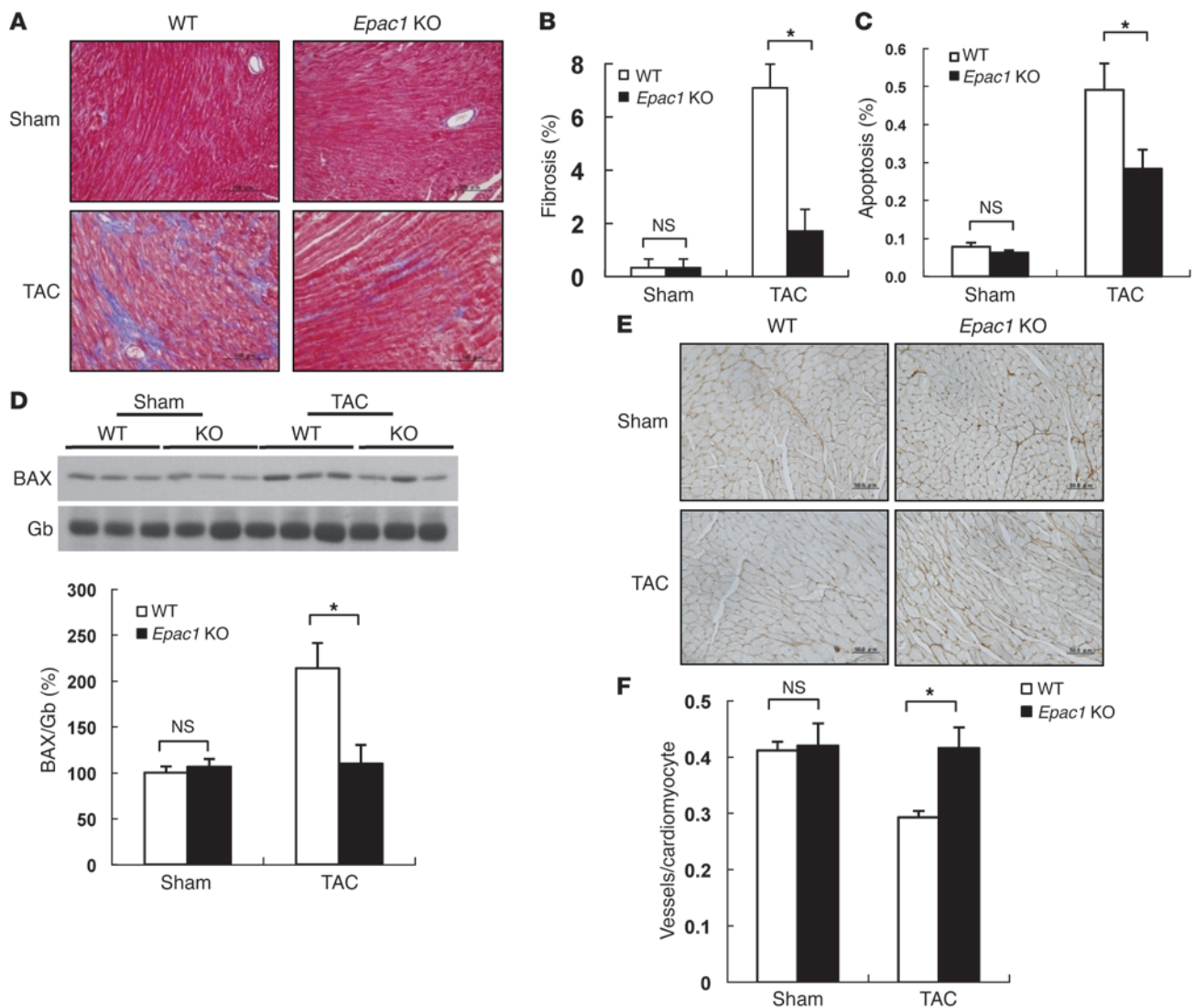


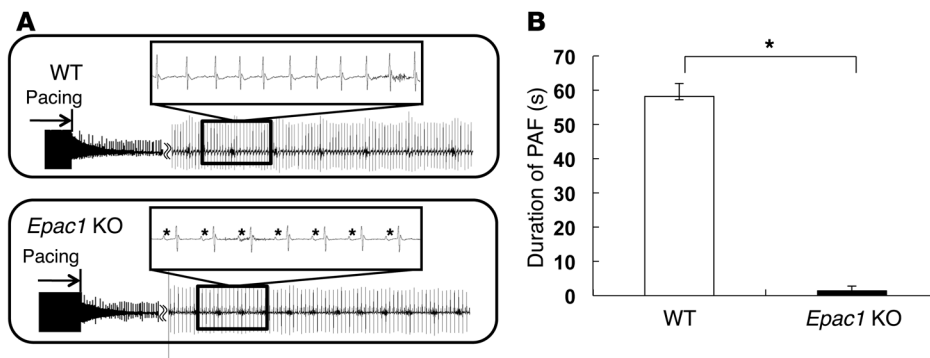
Figure 7

Accelerated morphological deterioration after aortic banding (TAC) was attenuated in *Epac1* KO. (A) Representative images of Masson-trichrome–stained sections of sham-operated and TAC-operated heart of WT and *Epac1* KO at 3 weeks. Scale bars: 100 μ m. (B) Quantitative analysis of the fibrotic area in sham-operated and TAC-operated heart at 3 weeks after TAC of WT and *Epac1* KO. Cardiac fibrosis was significantly increased after TAC in WT and *Epac1* KO, but magnitude of the increase was much smaller in *Epac1* KO ($n = 4$, $*P < 0.01$). (C) TUNEL-positive myocytes in LV myocardium were counted in WT and *Epac1* KO and expressed as percentage of total myocytes. Number of TUNEL-positive myocytes was significantly smaller in *Epac1* KO than in WT at 3 weeks after aortic banding ($n = 4-6$, $*P < 0.05$). (D) Expression of BAX protein was compared between WT and *Epac1* KO. Protein expression of BAX was determined by Western blot analysis, which showed greater expression in WT than in *Epac1* KO at 3 weeks after TAC ($n = 4$, $*P < 0.05$). Expression of BAX protein in the heart of sham-operated control WT was taken as 100%. Representative immunoblotting results are shown. (E) Representative images of double-immunostaining for dystrophin (brown) and PECAM (blue) of WT and *Epac1* KO at baseline and 3 weeks after aortic banding. Scale bars: 50 μ m. (F) Number of microvessels per cardiomyocyte was compared in WT and *Epac1* KO at baseline and at 3 weeks after aortic banding ($n = 4$, $*P < 0.01$).

KO (WT versus *Epac1* KO: from 9091 ± 240 to 6359 ± 393 mmHg [$P < 0.01$] versus from 7246 ± 226 to 6811 ± 250 mmHg, $n = 13-17$) and the magnitude of the decrease was much smaller in *Epac1* KO (Supplemental Figure 14, A and D). Min dP/dt was significantly increased in WT, while it remained unchanged in *Epac1* KO (WT versus *Epac1* KO: from -9110 ± 240 to -4389 ± 469 versus from -6201 ± 125 to -5131 ± 334 mmHg, $n = 13-18$, $P = \text{NS}$) and the magnitude of the increase was much smaller in *Epac1* KO (Supplemental Figure 14, B and E). End-diastolic pressure (EDP) was significantly increased in both

WT and *Epac1* KO (WT versus *Epac1* KO: from 7.2 ± 0.3 to 16.8 ± 1.4 versus from 6.8 ± 0.3 to 11.5 ± 1.1 mmHg, $n = 13-22$, $P < 0.01$), but the magnitude of the increase was significantly smaller in *Epac1* KO (Supplemental Figure 14, C and F).

We also examined the effect of long-term chronic pressure overload on cardiac hypertrophy and cardiac function at 5 weeks after banding in *Epac1* KO (Supplemental Figure 15A). At 5 weeks after banding, cardiac hypertrophy was increased in both WT and *Epac1* KO and the magnitude of the increase was similar (WT versus *Epac1*

**Figure 8**

The duration of AF induced by transesophageal rapid atrial pacing was decreased in *Epac1* KO. (A and B) Transesophageal pacing was performed at a cycle length of 30 ms for 1 minute, and the duration of pacing-induced AF was examined in *Epac1* KO and WT (A). The duration of AF was significantly decreased in *Epac1* KO compared with WT controls. AF was induced in all WT mice and its duration was 59 ± 3.4 seconds ($n = 4$). In *Epac1* KO, we found that AF was hardly inducible (1.4 ± 1.0 seconds, $n = 5$, $**P < 0.01$ versus WT) (B).

KO, LV/tibia length ratio: 7.7 ± 0.4 versus 7.7 ± 0.2 mg/mm; LV/body weight ratio: 4.4 ± 0.3 versus 4.6 ± 0.2 mg/g, $n = 15$, $P = \text{NS}$) (Supplemental Figure 15, B and C). However, cardiac function was significantly decreased in WT ($P < 0.01$), while it remained unchanged in *Epac1* KO (from $60\% \pm 1.1\%$ to $61\% \pm 0.7\%$, $n = 15$). LVESD was increased significantly in WT at 5 weeks after banding ($P < 0.01$), while it remained unchanged in *Epac1* KO (WT versus *Epac1* KO: from 2.9 ± 0.04 to 3.3 ± 0.1 versus from 3.3 ± 0.1 to 3.2 ± 0.1 mm, $n = 15$ –31) (Supplemental Figure 15, D–F). These results indicate that *Epac1* KO developed hypertrophy similar to that of WT in response to pressure overload, but did not develop cardiac failure.

Cardiac fibrosis and apoptosis are attenuated in Epac1 KO. There was no difference in degree of fibrosis between WT and *Epac1* KO at baseline. Thus, the development of cardiac fibrosis was unaffected in *Epac1* KO (WT versus *Epac1* KO: $0.3\% \pm 0.09\%$ versus $0.3\% \pm 0.06\%$, $n = 5$, $P = \text{NS}$). Aortic banding increased the area of fibrosis in both WT and *Epac1* KO (Figure 5, D and E), but the magnitudes of increase were much smaller in *Epac1* KO (WT versus *Epac1* KO: $7.1\% \pm 0.9\%$ versus $1.7\% \pm 0.8\%$, $n = 4$, $P < 0.01$) (Figure 7, A and B).

Similarly, there was no difference in the number of TUNEL-positive cells between WT and *Epac1* KO at baseline (WT versus *Epac1* KO: $0.08\% \pm 0.01\%$ versus $0.06\% \pm 0.007\%$, $n = 6$, $P = \text{NS}$). Aortic banding increased the number of TUNEL-positive cells in both WT and *Epac1* KO, but the magnitudes of increase were much smaller in *Epac1* KO (WT versus *Epac1* KO: $0.49\% \pm 0.07\%$ versus $0.24\% \pm 0.05\%$, $n = 4$ –6, $P < 0.05$) (Figure 7C). To examine changes in the molecules involved in apoptosis signaling, we quantitated BAX protein, an accelerator of apoptosis (31), in WT and *Epac1* KO and found that its expression after banding was significantly increased, by 2.1-fold in WT, but not in *Epac1* KO ($n = 4$ –6, $P < 0.05$) (Figure 7D).

Myocardial blood flow is preserved in Epac1 KO. We next examined cardiac response to intravenous ISO infusion, which is a hallmark of transmural myocardial blood flow, at 3 weeks after aortic banding (47). The increase of LVEF and decrease of LVESD in response to ISO were blunted in WT, but they were preserved in *Epac1* KO ($n = 4$ –5, $P < 0.01$ versus WT), suggesting that cardiac angiogenesis during aortic banding might be preserved in *Epac1* KO (Figure 6, E and F, and ref. 48).

We also examined the number of microvessels per cardiomyocyte. There was no difference in the number of microvessels between WT and *Epac1* KO at baseline (WT versus *Epac1* KO: 0.41 ± 0.02 versus 0.42 ± 0.04 , $n = 4$, $P = \text{NS}$). However, the number was significantly decreased in WT compared with *Epac1* KO at 3 weeks after aortic banding (WT versus *Epac1* KO: 0.29 ± 0.01 versus 0.42 ± 0.04 , $n = 4$, $P < 0.01$) (Figure 7, E and F).

Disruption of EPAC1 results in resistance to long-term ISO infusion. We also examined the effect of long-term ISO infusion on LV function (Supplemental Figure 16A). At 1 week after chronic ISO infusion, LVEF was significantly decreased in WT ($P < 0.01$), while it remained

unchanged in *Epac1* KO (WT versus *Epac1* KO: from 70 ± 0.8 to 60 ± 1.1 versus from $62\% \pm 1.4\%$ to $60\% \pm 0.9\%$, $n = 14$ –31).

Cardiac fibrosis after long-term ISO infusion was also significantly decreased in *Epac1* KO (WT versus *Epac1* KO: $4.6\% \pm 1.6\%$ versus $1.6\% \pm 0.2\%$, $n = 5$, $P < 0.01$) (Supplemental Figure 16, B and C). Similarly, cardiac apoptosis was also significantly attenuated in *Epac1* KO after long-term ISO infusion (WT versus *Epac1* KO: $0.49\% \pm 0.07\%$ versus $0.24\% \pm 0.05\%$, $n = 4$ –5, $P < 0.05$) (Supplemental Figure 16D).

The degree of cardiac hypertrophy was increased at 1 week, but remained similar in *Epac1* KO and WT (LV/tibial length ratio for WT versus *Epac1* KO: 5.7 ± 0.2 versus 5.9 ± 0.1 , $n = 14$ –15, $P = \text{NS}$; LV/BW ratio for WT versus *Epac1* KO: 3.5 ± 0.2 versus 3.7 ± 0.1 , $n = 14$ –15, $P = \text{NS}$) (Supplemental Figure 17, A and B).

Silencing of EPAC1 in cardiac fibroblasts did not alter cell proliferation. Since the mouse model is a global *Epac1* KO, the phenotype of less fibrosis after long-term ISO infusion or aortic banding may result from altered cell proliferation or signaling in cardiac fibroblasts. We thus examined the effect of silencing EPAC1 on neonatal rat cardiac fibroblast proliferation in response to ISO, using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Supplemental Figure 9B, Supplemental Figure 18, and ref. 49). Cardiac fibroblasts were cultured in medium with 10% fetal bovine serum for 24 hours and were starved for 48 hours without serum prior to stimulation. At 24 hours after ISO (1 μM or 10 μM) stimulation, MTT assay was performed. Cell proliferation was significantly increased ($P < 0.01$) by ISO (10 μM) from baseline (control siRNA versus EPAC1 siRNA: from $100\% \pm 5.7\%$ to $137\% \pm 4.6\%$ versus from $108\% \pm 1.7\%$ to $140\% \pm 4.6\%$, $n = 6$ –8), but the magnitudes of the increase were similar ($P = \text{NS}$) in cells transfected with control or EPAC1 siRNA (ref. 49 and Supplemental Figure 18A).

We also examined p44/42 MAPK (ERK1/2) phosphorylation on tyrosine-202/threonine-204 and SRC phosphorylation on tyrosine-416 because phosphorylations of ERK1/2 and SRC are important for G protein-coupled receptor-mediated cardiac fibroblast proliferation (50). ERK1/2 phosphorylation and SRC phosphorylation were similar in both control and EPAC1 siRNA-treated cells (Supplemental Figure 18, B–D). However, both ERK1/2 and SRC phosphorylations were significantly ($P < 0.01$) increased in response to ISO (10 μM) in cells transfected with control or EPAC1

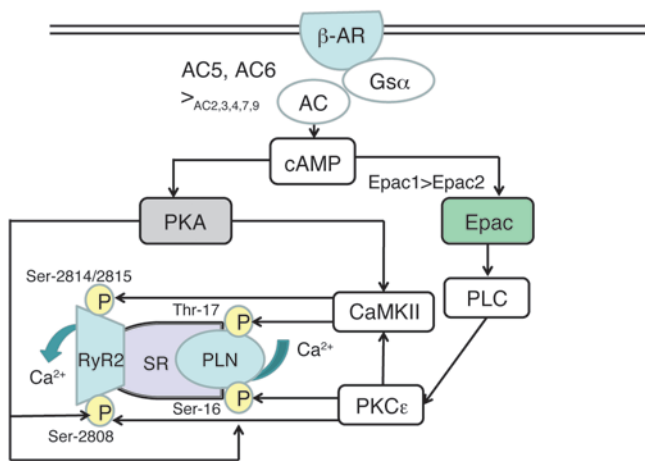


Figure 9

A schematic model of cAMP/EPAC signaling as opposed to cAMP/PKA signaling in the heart. Ca²⁺ stored in the SR is released into the cytosol to activate cardiac muscle contraction and subsequently reaccumulated to promote relaxation. PLN phosphorylation on serine-16 as well as threonine-17 occurs via the EPAC/PLC/PKCε/CaMKII pathway (27, 28). However, under physiological conditions, PLN phosphorylation on serine-16 by PKA rather than on threonine-17 by CaMKII is the major regulator of Ca²⁺ cycling in the heart (56, 57). Our current study indicates that PLN on serine-16 and RyR2 on serine-2808 are phosphorylated by EPAC1 in addition to and independently of PKA or CaMKII. More importantly, hyperphosphorylation of PLN on serine-16 was recently reported to be associated not only with an increase in cardiac function in young animals (16, 17), but also with arrhythmia and cardiomyopathy after adrenergic stress, aortic banding, or ischemia (18, 20, 21). Our results suggest that *Epac1*-mediated hyperphosphorylation of PLN and RyR2 might be required for the development of heart failure as well as arrhythmia, in addition to PKA- or CaMKII mediated activation.

siRNA, and the magnitudes of their increase were similar in the 2 cases (ERK1/2: control siRNA versus EPAC1 siRNA: from 100 ± 2.5 to 151 ± 2.0 versus from 108% ± 3.7% to 154% ± 6.7%, *n* = 4; SRC: control siRNA versus EPAC1 siRNA: from 100% ± 2.5% to 233% ± 12% versus from 107% ± 9.7% to 235% ± 23%, *n* = 7-8). These data indicate that silencing EPAC1 in cardiac fibroblasts did not alter the cell-proliferative response to β-AR-signaling stimulation.

Disruption of EPAC1 results in resistance to aging-related cardiomyopathy. Key features of aging-related cardiomyopathy were examined, i.e., LV weight/tibial length ratio, LV function, apoptosis, and fibrosis. The LV weight/tibial length ratio was not different between old WT (5.6 ± 0.5) and old *Epac1* KO (5.6 ± 0.5) at baseline (24–32 months). However, aging-related changes of cardiac function (LVEF in WT versus *Epac1* KO: from 70% ± 0.8% [young] to 58% ± 1.3% [old] [*P* < 0.01] versus from 60% ± 1.1% to 58% ± 2.2%, *P* = NS) (Supplemental Figure 16E), fibrosis (old WT versus old *Epac1* KO: 3.2% ± 0.2% versus 1.2% ± 0.2%, *n* = 5–8, *P* < 0.01) (Supplemental Figure 16F) and apoptosis (old WT versus old *Epac1* KO: 0.38% ± 0.04% versus 0.22% ± 0.03%, *n* = 5, *P* < 0.05) (Supplemental Figure 16G) were all significantly decreased in *Epac1* KO.

Incidence of atrial fibrillation after rapid atrial pacing is attenuated in Epac1 KO. It has been reported that phosphorylations of RyR2 on serine-2814 and PLN on threonine-17 are increased after rapid atrial pacing in atrial myocytes and genetic inhibition of RyR2 phosphorylation on serine-2814 attenuates the incidence of atrial

fibrillation (AF) induced by rapid atrial pacing (51). Accordingly, we examined the effects of *Epac1* deletion on the initiation and maintenance of AF in the mouse model (Figure 8 and ref. 52). Transesophageal rapid atrial pacing was performed at a cycle length of 30 ms for 1 minute, followed by measurement of the duration of pacing-induced AF (Figure 8A). In all WT control mice, AF was consistently induced for 50 to 60 seconds. In striking contrast, little (less than 4 seconds) or no AF was induced in *Epac1* KO. These data indicate that *Epac1* deletion may suppress the initiation and maintenance of AF after transesophageal rapid atrial pacing, probably through inhibition of hyperphosphorylation of RyR2 and/or PLN in atrial myocytes (AF duration for WT versus *Epac1* KO: 58 ± 3.8 versus 1.4 ± 1.4 seconds, *n* = 4–5, *P* < 0.01).

Catecholamine-mediated spontaneous activity in the pulmonary vein cardiomyocytes is attenuated in Epac1 KO. High-frequency focal activity in the pulmonary vein cardiomyocytes may contribute to arrhythmic activity, which is induced mostly via an increase of intracellular Ca²⁺ derived from SR (53, 54). The incidence of the spontaneous activity was not different in WT (4/17:24%) and *Epac1* KO (7/24:29%) at baseline (*P* = NS) (Supplemental Figure 19B). However, it was increased after noradrenaline (1 μM) treatment in both WT and *Epac1* KO, though its incidence was less in *Epac1* KO (12/17:71%) than in WT (15/15:100%) (*P* < 0.05 evaluated by Fisher's exact probability test). These findings indicated that catecholamine-mediated spontaneous activity in the pulmonary vein cardiomyocytes is attenuated in *Epac1* KO (Supplemental Figure 19, A and C).

Disruption of EPAC2 does not prevent the cardiac hypertrophy and the development of heart failure in response to pressure overload stress. In order to examine the effects of EPAC2, another cardiac isoform of EPAC, on the development of heart failure, we generated *Epac2* KO (Supplemental Figure 20). *Epac2* KO did not show any change in the size of the heart or in cardiac function, despite the smaller body weight, compared with that in WT (Supplemental Table 1). We examined PLN phosphorylation on serine-16 and on threonine-17 in *Epac2* KO, but the levels were similar in both WT and *Epac2* KO (Supplemental Figure 21).

In order to examine the role of EPAC2 in the development of heart failure, we performed aortic banding in *Epac2* KO and WT for 3 weeks. At baseline, there was no difference between WT and *Epac2* KO in the LV weight (mg)/tibial length (mm) (LV/tibial length ratio in WT versus *Epac2* KO: 5.7 ± 0.1 versus 5.2 ± 0.2 mg/mm, *n* = 5–7, *P* = NS). The degree of cardiac hypertrophy was significantly (*P* < 0.01) increased at 3 weeks, but the magnitudes of the increase were similar in both WT and *Epac2* KO (LV/tibial length ratio in WT versus *Epac2* KO: 7.1 ± 0.4 versus 6.8 ± 0.2, *n* = 4, *P* = NS) (Supplemental Figure 22A).

At 3 weeks after banding, cardiac function (LVEF) was significantly decreased in both WT and *Epac2* KO from baseline (WT versus *Epac2* KO: from 73% ± 1.1% to 55% ± 3.1% [*P* < 0.01, *n* = 4–5] versus from 72% ± 1.1% to 58% ± 5.2% [*P* < 0.05, *n* = 5–7]), but the magnitudes of the decrease were similar (*P* = NS) (Supplemental Figure 22B). LVESD was increased in both WT and *Epac2* KO at 3 weeks after banding (WT versus *Epac2* KO: from 2.7 ± 0.07 to 3.1 ± 0.2 [*P* < 0.05, *n* = 4–5] versus from 2.6 ± 0.05 to 3.1 ± 0.2 mm [*P* < 0.05, *n* = 4–13]), but the magnitudes of the increase were similar (*P* = NS) (Supplemental Figure 22C).

There was no difference in the degree of fibrosis between WT and *Epac2* KO at baseline (Supplemental Figure 22, D and E). Thus, the development of cardiac fibrosis was unaffected in *Epac2* KO (WT versus *Epac2* KO: 0.4% ± 0.08% versus 0.4% ± 0.09%, *n* = 5,



$P = \text{NS}$). Aortic banding increased the area of fibrosis in both WT and *Epac2* KO, but the magnitudes of the increase were similar (WT versus *Epac2* KO: $22\% \pm 3.6\%$ versus $18\% \pm 5.3\%$, $n = 4$, $P = \text{NS}$) (Supplemental Figure 22, D and E).

Similarly, there was no difference in the number of TUNEL-positive cells between WT and *Epac2* KO at baseline (WT versus *Epac2* KO: $0.09\% \pm 0.02\%$ versus $0.09\% \pm 0.01\%$, $n = 5$, $P = \text{NS}$) (Supplemental Figure 22F). Aortic banding significantly ($P < 0.01$) increased TUNEL-positive cells in both WT and *Epac2* KO, but the magnitudes of increase were similar (WT versus *Epac2* KO: $0.8\% \pm 0.04\%$ versus $0.8\% \pm 0.05\%$, $n = 4-5$, $P = \text{NS}$) (Supplemental Figure 22F). These results indicate that *Epac2* KO did not show decreased cardiac contractility and PLN phosphorylation at baseline and did not show resistance after aortic banding, indicating that the cardioprotective effects of EPAC under stress such as pressure overload (Figures 5-7, Supplemental Figure 14, and Supplemental Figure 15), chronic catecholamine stress (Supplemental Figure 16, A-D), aging stress (Supplemental Figure 16, E-G), and AF susceptibility (Figure 8) are *Epac1* KO-specific phenotypes.

Discussion

RyR2 is a prominent regulator of systolic contraction and PLN is a prominent regulator of myocardial diastolic relaxation via modulation of the activity of the SERCA2a. Specifically, RyR2 in its phosphorylated state following the Ca^{2+} influx through the L-type Ca^{2+} channel is an accelerator of Ca^{2+} leakage from SR, and PLN in its dephosphorylated state is an inhibitor of Ca^{2+} transport to SR via SERCA2a, while phosphorylation of PLN removes this inhibition, indicating that both molecules have central roles in modulating Ca^{2+} homeostasis and, therefore, cardiac function (10, 11, 46).

Early studies of enhanced SR Ca^{2+} leakage in heart failure focused on PKA, a classic cAMP target. Recently, it has been shown that β -AR stimulation can also induce PKA-independent, i.e., CaMKII- or EPAC-mediated SR Ca^{2+} leakage in vitro by increasing RyR2 phosphorylation on serine-2814/2815 and PLN phosphorylation on threonine-17 via PLC ϵ /PLC ζ /CaMKII signaling (27-30, 55). Nevertheless, key functional issues remain to be clarified because most previous studies relied on pharmacological stimulation with 8-CPT, an EPAC-selective but not isoform-selective cAMP analogue, which could have off-target effects (37). First, it is unknown whether these effects are mediated by EPAC1 or EPAC2. Second, it is unknown whether EPAC-regulated Ca^{2+} homeostasis is important for cardiac function and arrhythmogenesis under physiological conditions. Third, it remains unknown whether EPAC regulates PLN phosphorylation on serine-16 in addition to threonine-17, which is known to be a major regulator of Ca^{2+} cycling in the heart and closely associated not only with an increase in cardiac function (16, 17, 56, 57), but also with arrhythmia and cardiomyopathy after chronic catecholamine infusion, aortic banding, or ischemia (18, 20, 21). We thus generated *Epac1* KO and *Epac2* KO in addition to silencing EPAC1 with siRNA and selectively inhibiting PKA with Ad-PKI-GFP in cardiac myocytes. The results clearly demonstrated that EPAC1-regulated Ca^{2+} homeostasis via PLN phosphorylation on serine-16, together with RyR2 phosphorylation on serine-2808 and serine-2814, may cause transition from hypertrophy to heart failure and evoke susceptibility to arrhythmia (Figure 9 and refs. 21, 58).

Pln-null mice show no gross developmental abnormalities, but exhibit enhanced myocardial contractility owing to increased SERCA2a pump activity and higher SR Ca^{2+} load in cardiomyo-

cytes (12). Ablation of PLN restored contractility in some cardiomyopathic mouse models, such as cardiac-specific calsequestrin overexpression, targeted disruption of muscle-specific LIM protein, or cardiac-specific β_1 -AR overexpression (16, 17, 59). Thus, various therapeutic approaches targeting or inhibiting PLN have been examined to rescue the failing heart (60, 61).

However, it was subsequently reported that ablation of PLN shows no cardioprotective effect against chronic pressure overload or ischemic injury and, indeed, exacerbates cardiac dysfunction in mice with cardiac CaMKII δ c overexpression, even if the Ca^{2+} transient is improved (19). More recently, it was reported that hyperphosphorylation of PLN in mice with constitutively active phosphatase inhibitor-1 increased contractile function in young animals, but also resulted in increased susceptibility to arrhythmias and cardiomyopathy in response to chronic catecholamine stress and aging, probably through increased Ca^{2+} leakage, leading to arrhythmogenicity and cardiomyocyte apoptosis (21). Our current data obtained in *Epac1* KO and *Epac2* KO demonstrated that EPAC1 plays an important role in PLN phosphorylation on serine-16 and EPAC1-mediated PLN phosphorylation rather than PKA-mediated PLN phosphorylation might have a pivotal role in the development of heart failure in response to chronic pressure overload, catecholamine stress, and aging, because the PKA pathway remains intact in *Epac1* KO.

For many decades, it has been believed that the major target of catecholamine/cAMP signaling is PKA. Although acute sympathetic stimulation and activation of the cAMP/PKA pathway represent a major mechanism of improvement of cardiac function, previous studies by us and other groups using transgenic models have demonstrated that chronic activation of these pathways arising from specific cardiac overexpression of β -AR, Gs α , and PKA results in cardiomyopathy (3-5). On the other hand, disruption of type 5 AC, a major cardiac AC isoform, protects the heart from chronic pressure overload and catecholamine stress without affecting baseline cardiac function in mice (6-8). Interestingly, our results indicate that cardiac function is preserved in response to chronic pressure overload, chronic catecholamine, and aging stress in *Epac1* KO, indicating that both phosphorylation of PLN on serine-16 and phosphorylation of RyR2 on serine-2808 and serine-2814 by EPAC1, rather than PKA, might be essential for the transition of hypertrophy to heart failure in response to chronic activation of β -AR signaling (11, 46, 58, 62).

Increased Ca^{2+} leakage in atrial myocytes has been demonstrated to be a cause and consequence of AF, but the mechanism remains poorly understood (63). Recently, it was demonstrated that PLN phosphorylation on serine-16, rather than threonine-17, was significantly increased in the atrium of chronic AF patients while PKA phosphorylation of inhibitory troponin subunit was unaltered (22). Similarly, it was demonstrated that rapid atrial pacing induces phosphorylation of RyR2 on serine-2814 and PLN on threonine-17 and genetic inhibition of RyR2 phosphorylation on serine-2814 prevents AF after rapid atrial pacing (64). These results indicate that altered Ca^{2+} homeostasis in atrial myocytes through EPAC-mediated hyperphosphorylation of PLN and RyR2 may cause susceptibility to AF. We thus hypothesized that EPAC1 plays an important role in the occurrence of AF, and we confirmed this idea using a transesophageal pacing-induced AF mouse model. American College of Cardiology, American Heart Association, and European Society of Cardiology guidelines strongly recommend β -blockers for the management of adrenergically induced AF, but it is difficult



to use β -blockers in patients with chronic obstructive pulmonary disease or severe heart failure. Therefore, EPAC may be available as a new target of pharmacotherapy for adrenergically induced AF (65).

PLN was demonstrated to be phosphorylated by PKC in vitro in addition to PKA and CaMKII (39, 41), but the mechanism of PKC-dependent PLN phosphorylation remains unclear and its importance for cardiac function is controversial (66, 67). Our current in vitro studies, in agreement with earlier studies (27–29), indicate that EPAC regulates PLN phosphorylation on both serine-16 and threonine-17 through PLC/PKC ϵ signaling. However, our in vivo studies using *Epac1* KO show that EPAC1 regulates PLN phosphorylation on serine-16 at baseline and under stress, but it does not affect the phosphorylation on threonine-17. Previous in vitro studies have shown that serine-16 of PLN and threonine-17 of PLN can be readily and independently phosphorylated by PKA and CaMKII, respectively. However, previous in vivo studies indicated that PLN phosphorylation on threonine-17 can be induced by β -AR stimulation that is sufficient to increase cytosolic Ca²⁺ concentration, which in turn would activate CaMKII and/or inhibit the phosphatase that dephosphorylates PLN. The apparent difference between in vitro and in vivo PLN phosphorylation is yet to be reconciled (68), but a possible explanation of the difference between our in vitro and in vivo data on PLN phosphorylation at threonine-17 is that the extent of CaMKII activation was not enough to produce a significant difference between WT and *Epac1* KO under β -AR stimulation in response to ISO in the Langendorf-perfused heart system used in this study. We also cannot exclude the possibility that this might be due to a difference in the specificity of the gene-silencing technique in vitro with siRNA and in vivo with genetic KO because complete gene silencing can be achieved by genetic KO (31), but not by using siRNA (Supplemental Figure 9 and ref. 69).

In contrast with *Epac1* KO, disruption of EPAC2 does not alter the cardiac function or PLN phosphorylation at baseline and has no protective effects after aortic banding. These data indicate that EPAC1, but not EPAC2, is a major regulator of cardiac function at baseline and under stress.

More importantly, cardiac function and the Ca²⁺ transient are similar in global *Plec* KO and WT at baseline even if the ISO-stimulated Ca²⁺ transient is smaller in *Plec* KO (70). Global *Plec* KO does not show a protective phenotype against chronic catecholamine stress (70) in contrast with *Epac1* KO. Otherwise, global *Plec* KO is more susceptible than WT to the development of cardiac hypertrophy and fibrosis after chronic ISO infusion (70). In contrast with global *Plec* KO, myocyte-specific *Plec* KO is not susceptible, but is rescued from cardiac hypertrophy as well as cardiac dysfunction after TAC (71). Also, PLC ϵ was demonstrated to generate a multifunctional complex with muscle-specific A-kinase-anchoring protein at or near the nuclear envelope along with *Epac1*, PKC ϵ , PKD, and RyR2 in cardiac myocytes (71). *Pkc* KO does not show a protective phenotype and is not susceptible to development of cardiac hypertrophy after TAC, in contrast with global *Plec* KO and myocyte-specific *Plec* KO (72). These data indicate that disruption of EPAC1 modulates or antagonizes the downstream PLC ϵ /PKC ϵ pathway, which in turn is associated with the cardioprotective phenotype of *Epac1* KO under stress.

During the revision of this manuscript, another group reported that cardiac function was not different between WT and *Epac1* KO at baseline in contrast with our findings. The reason for the difference of the cardiac phenotype between our *Epac1* KO and that generated by the other group is not clear (30). However, the genetic back-

grounds of *Epac1* KO generated by us (CBA-C57BL/6) and by the other group (R1-C57BL/6) were different, and this might cause the difference in the mouse phenotype (73, 74), as in the case of type 5 AC KO (129/SvJ-C57BL/6) generated by us (6, 7) and another group (C57BL/6) (75) and *Plec* KO generated by 2 different groups: 129/Sv-C57BL/6 (76) versus 129/S6-C57B/6 (70). Also, environmental influences have been reported to alter phenotype, as in the case of *Tgfb* KO (77, 78), and we cannot exclude this as another possibility.

In summary, we have demonstrated that disruption of EPAC1 protects the heart from chronic pressure overload, chronic catecholamine stress, aging-related cardiomyopathy, and AF susceptibility through the inhibition of PLN phosphorylation on serine-16 in addition to the inhibition of RyR2 phosphorylation on serine-2808 and serine-2814. The cAMP/PKA pathway is believed to be a major pathway for the development of heart failure as well as arrhythmia (5, 21). Our present data show that the cAMP/EPAC1 pathway plays an important role independently of the cAMP/PKA pathway in the development of heart failure and arrhythmia.

Methods

Reagents. All chemicals were obtained from Sigma-Aldrich, except 8-CPT-AM (Biolog Life Science Institute) and Indo-1/AM (Dojindo Laboratories).

Mice. Generation of *Epac1* KO has been previously reported (31). *Epac2* KO were similarly generated by means of homologous recombination. Briefly, the targeting vector was constructed by inserting pENTR loxP/PGK-Neo-pA/loxP (Laboratory for Animal Resources and Genetic Engineering [LARGE]; <http://www.cdb.riken.jp/arg/cassette.html>) into exon 1 and intron 1 of the genomic *Epac2* locus (Supplemental Figure 20A). The targeting vector was introduced into TT2 embryonic stem cells (79), and homologous recombinant clones were first identified by PCR, then confirmed by Southern blot analysis (Supplemental Figure 20B and refs. 6, 31). The targeted embryonic stem cell clones were injected into CD 18-cell-stage embryos, and the resultant male chimeras were mated with C57BL/6 females to establish germ line transmission. All experiments were performed on C57BL/6 and CBA mixed background 3- to 5-month-old male homozygous *Epac2* KO and WT littermates from F1 heterozygote crosses. Mice were genotyped by PCR using a mixture of 2 primer sets (F1, TGGGTGGGTGGTTTCCAATG; B1, CCTAACACAGACCTTGAGAGAGCG; F2, TCGTGCTTTACGGTATCGCCGCTCCCGATT; B2, CGTAGCTCCAATCCTTCCATTCA). The PCR conditions consisted of 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds each, 60°C for 30 seconds, and 72°C for 30 seconds, followed by 72°C for 7 minutes (Supplemental Figure 20C). mRNA expression of the *Epac* isoforms (*Epac1* and *Epac2*) in the heart of *Epac2* KO was examined by Northern blot analysis (Supplemental Figure 20D and ref. 80).

All experiments were performed on 3- to 6-month-old homozygous KO (*Epac1*, *Epac2*) mice and WT littermates.

Western blotting. Hearts were removed from each animal or Langendorf apparatus, snap-frozen in liquid nitrogen, and stored at –80°C after physiological studies. Crude cardiac membrane fraction or whole -issue homogenate was prepared, separated on 6% to 15% SDS-polyacrylamide gel and blotted onto nitrocellulose membrane. Western blotting was conducted using commercially available antibodies. β_1 -AR, β_2 -AR, β ARK, G β , Gq, type 5/6 AC (AC5/6), and PKA-catalytic subunit antibodies were purchased from Santa Cruz Biotechnology Inc. G α and G β antibodies were purchased from PerkinElmer. PLN, PLN phosphorylated on serine-16 and threonine-17, RYR2, and RYR2 on serine-2808 and serine-2814 were purchased from Badrilla. Antibodies to PKA regulatory subunits (RII α and RI α) were purchased from BD Transduction Laboratories. EPAC1, EPAC2, CaMKII, phospho-CaMKII (threonine-286), ERK1/2, phospho-ERK1/2



(tyrosine-202/threonine-204), SRCc, and phospho-SRC (tyrosine-416) antibodies were purchased from Cell Signaling Technology. SERCA2a antibody was purchased from Thermo Scientific. Expression of proteins was quantified by densitometry.

Real-time quantitative PCR. Total RNA was extracted using TRIzol Reagent (Life Technologies) according to the manufacturer's protocol. RNA concentration and quality were determined by spectrophotometry. To remove contaminating genomic DNA, samples were treated with DNase I (Life Technologies). Total RNA was reverse-transcribed with SuperScript First-Strand Synthesis System for RT-PCR kit (Life Technologies) according to the manufacturer's instructions.

mRNA expression of *Pkca*, *Pkcb*, *Pkcd*, *Pkcg*, and *Pkce* was quantified by quantitative real-time PCR using the ABI-PRISM 7700 Sequence Detection System with SYBR Green. The primer pairs for each subtype of PKC were as follows:

Pkca, forward, 5'-GCCGAGTGTCTTATGAAAGTA-3', reverse, 5'-GCTCCATGTGTGCCATTCAATTAG-3'; *Pkcb*, forward, 5'-AAGACATTCTGTGGCACTCCAGAC-3', reverse, 5'-AGCCAACATTTATACAGCAGGAC-3'; *Pkcd*, forward, 5'-CAAAGGCCGCTTCGAACTCTAC-3', reverse, 5'-GGCCATCCTTGTCCAGCATTAC-3'; *Pkcg*, forward, 5'-CACAGACTTCGGCATGTGTAAGA-3', reverse, 5'-CCATAGGGCTGATAGGCAAATGA-3'; and *Pkce*, forward, 5'-ATGGCGTGACAACCTACCACCTTC-3', reverse, 5'-CCGGCCATCATCTCGTACATC-3'. The amount of each subtype mRNA was normalized to that of 18S rRNA to obtain the relative amount.

Neonatal cardiac myocyte/fibroblast preparation and transfection of siRNA. Primary cultures of neonatal rat or mouse cardiomyocytes or fibroblast were prepared (81–83). Double-stranded siRNA to the selected region of each subtype of PKC (α , β , ϵ , δ , γ), EPAC1, and negative siRNA (control) were purchased from QIAGEN. At 48 hours after plating, cultured neonatal rat cardiac myocytes/fibroblasts (1.2×10^5 cells per 24-mm plate) were transfected with siRNA using Lipofectamine RNAiMAX (Life Technologies), according to the manufacturer's instructions.

Briefly, siRNA (20 nM) in 50 μ l of OptiMEM I medium (Life Technologies) and 0.5 μ l of Lipofectamine RNAiMAX (Life Technologies) in 50 μ l OptiMEM I medium were mixed and then added to the dishes. At 24 hours after transfection, the culture medium (minimum essential medium) was replaced with fresh medium and incubation was continued for 24 hours in a humidified atmosphere of 5% CO₂ in air at 37°C. ISO treatment was performed at 1 μ M (mouse) or 10 μ M (rat), which was chosen based on preliminary experiments performed with reference to previous studies on PLN phosphorylation (70, 84). The efficiency of knockdown of each subtype of PKC was evaluated at 24 to 48 hours after transfection by means of real-time quantitative PCR. Negative siRNA used as a control was purchased from QIAGEN (Cat no. 1027281). The siRNA sequences of PKC subtype were as follows: PKC α , forward, 5'-GAAGGGUUCUCGUAUGUCATT-3', reverse, 5'-UGACAUACGAGAACCCUCAA-3'; PKC β , forward, 5'-GAAGAAGGCGAGUACUUUATT-3', reverse, 5'-UAAAGUACUCGCCUUCUUCCT-3'; PKC δ , forward, 5'-CCUUUAAGCCAAAGUGAATT-3', reverse, 5'-UUCACUUUGGGCUUAAAGGGC-3'; PKC γ , forward, 5'-GGGCGAGUAUACAAUGUATT-3', reverse, 5'-UACAUUGUAUACUCGCCCTC-3'; and PKC ϵ , forward, 5'-CGAUGAGUUCGUCACUGAUTT-3', reverse, 5'-AUCAGUACGAAACUCAUCGTG-3'.

Generation of adenovirus. A DNA oligo corresponding to the coding sequence for amino acids 1–25 (MTDVETTYADFIASGRTGRRNAIHD) of mouse protein kinase (cAMP-dependent, catalytic) inhibitor α (*Pkia*) (mouse Entrez Gene ID 18767) was synthesized. Recombinant adenovirus encoding PKI α -GFP infusion gene was generated using the AdEasy system (44).

Adult mouse ventricular myocytes preparation. Adult mouse ventricular myocytes were prepared from mice by Langendorf perfusion and collagenase digestion, as described (45, 85, 86).

Measurement of intracellular Ca²⁺. Intracellular Ca²⁺ was measured with Indo-1, as in our previous study (87). The cells were preincubated at 37°C with the acetoxymethyl derivative of Indo-1 (Indo-1/AM 10 μ M, 45 minutes) in the experimental chamber. After preincubation, the chamber was placed on a fluorescence microscope (Olympus IX70; Olympus Corp.) and perfused with HEPES-Tyrode solution as described above. Ca²⁺ transients were evoked at 1 Hz through platinum wire pairs with rectangular current pulses of 3 ms duration generated by an electrical stimulator (SEN-3303; Nihon Kohden). The cells were excited at 360 nm with an Xenon lamp, and the emission bands at 395 to 415 nm and 470 to 490 nm (Indo-1) were separated by means of dichroic mirrors (W-VIEW system; Hamamatsu Photonics) and detected with a high-speed cooled CCD camera (C6790; Hamamatsu Photonics) at a time resolution of 1.95 ms.

The ratio of emission between short-wavelength fluorescence and long-wavelength fluorescence was calculated (Aquacosmos software; Hamamatsu Photonics). In situ calibration of Indo-1 fluorescence ratio values to intracellular Ca²⁺ concentration was performed as described previously (87). The time constant (τ) for the Ca²⁺ transient decay was obtained by fitting the decay of the transients to a single exponential equation: $Y(t) = a \times \exp^{-k \times t} + b$, where a = Ca²⁺ transient amplitude; b = baseline value; k is the rate coefficient. k was obtained from the fitting, and τ was calculated as $\tau = 1/k$.

Measurement of contractile force. Myocardial contractile force was measured as previously described (88). RV free wall strips (approximate length and width: 4 and 2 mm, respectively) were rapidly isolated from mice anesthetized with ether. Preparations were placed horizontally in a 20 ml organ bath containing modified Ringer solution of the following composition: 135 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 15 mM NaHCO₃, and 5.5 mM glucose (pH 7.4 at 36°C). The solution was gassed with 95% O₂–5% CO₂ and maintained at 35–36°C. The preparations were driven by a pair of platinum plate electrodes (field stimulation) with rectangular current pulses (1 Hz, 3 ms, 1.5 \times threshold voltage) generated by an electronic stimulator. Developed tension was recorded isometrically with a force-displacement transducer connected to a minipolygraph.

Patch-clamp recording. The L-type Ca²⁺ channel current (I_{CaL}) was measured as described, with minor modifications (89, 90). Briefly, I_{CaL} was measured in the whole-cell patch-clamp configuration with EPC8 (HEKA) via an A/D converter, Digidata1440A, and Clampex 10.0 (Axon Instrument) at room temperature. Data were analyzed by Clampfit 10.0 (Axon Instrument) and GraphPad Prism 4 (GraphPad Software). Patch-pipettes were fabricated from borosilicate glass and had tip resistance of 1.2–2.2 M Ω when filled with pipette solution containing the following: 120 mM CsCl; 20 mM tetraethylammonium chloride; 5 mM adenosine 5'-triphosphate magnesium salt; 5 mM creatine phosphate disodium salt; 0.2 mM guanosine 5'-triphosphate; 14 mM EGTA; 10 mM HEPES; titrated to pH 7.3 with CsOH. The extracellular solution contained the following: 137 mM NaCl; 5.4 mM KCl, 10 mM HEPES, 1 mM MgCl₂, 10 mM glucose, 2 mM CaCl₂, 0.01 mM tetrodotoxin; titrated to pH 7.4 with NaOH. I_{K1} , the inward rectifier K⁺ current, was suppressed by the addition of BaCl₂ at 0.2 mM to the extracellular solution. The extracellular solution surrounding the patch-clamped cell was rapidly changed by a custom-made concentration clamp system (91). I_{CaL} was isolated as the current component blocked by CdCl₂ at 0.2 mM.

The Na⁺-Ca²⁺ exchange current (I_{NCX}) was measured with pipette solution containing the following: 50 mM CsOH; 10 mM Cs-methanesulfonate; 10 mM TEA-Cl; 20 mM NaCl; 5 mM Mg²⁺-ATP; 10 mM HEPES; 20 mM BAPTA; 10 mM CaCl₂ (226 nM free Ca²⁺); titrated to pH 7.3 with CsOH. Extracellular solution contained the following: 137 mM NaCl, 5.4 mM CsCl, 10 mM HEPES, 1 mM MgCl₂, 10 mM glucose, 2 mM CaCl₂, 0.01 mM tetrodotoxin, 0.2 mM BaCl₂, 10 mM 4-AP, 0.01 mM nitrendipine, 0.1 mM niflumic acid, 0.005 mM ryanodine; titrated to pH 7.4 with NaOH. In order to exclude the secondary effect of intracellular Ca²⁺ handling on I_{NCX} , the concentration of free



Ca²⁺ was buffered with a high concentration of BAPTA. *I_{NCX}* was recorded by applying ramp pulse ranging from 80 mV to -150 mV for 100 ms following step pulses to -40 mV for 40 ms and to 0 mV for 10 ms to inactivate *I_{Na}* and *I_{CaL}*, respectively, from the holding potential of -70 mV. *I_{NCX}* was isolated as the current component blocked by NiCl₂ at 5 mM.

Physiological studies. Mice were anesthetized with isoflurane vapor titrated to maintain the lightest anesthesia possible. On average, 1.5% vol/vol isoflurane vapor was required to maintain adequate anesthesia. Echocardiographic measurements and invasive hemodynamic measurements were performed (6–8, 92). Intravenous infusion of ISO (0 to 0.40 µg/kg/min for 5 minutes) was performed using an infusion pump (6, 8). Transverse aortic banding and long-term infusion of ISO were performed for 7 days at 60 mg/kg/d as described previously by us (7, 8). ECG recordings of awake, free-moving mice were obtained using a telemetric method (PhysioTel; Data Science International). AF was induced by transesophageal rapid atrial pacing in mice as described previously (52).

Histological analysis. Heart specimens were fixed with formalin, embedded in paraffin, and sectioned at 6-µm thickness. Interstitial fibrosis was evaluated by Masson trichrome staining using the Accustatin Trichrome Stain Kit (Sigma-Aldrich). DNA fragmentation was detected in situ by TUNEL staining as described previously (7, 8). Frozen cross sections of the heart samples were immunohistochemically double-stained by the use of antibodies against PECAM (BD Biosciences – Pharmingen) and dystrophin (Novocastra Laboratories). The number of microvessels per cardiomyocyte was calculated as described previously (48).

Proliferation assay. MTT cell viability assay was used to measure neonatal rat cardiac fibroblast proliferation using Cell Proliferation Kit I according to the manufacturer's instructions (Roche) (49). Briefly, cardiac fibroblast were seeded onto 96-well tissue culture plates (1 × 10³ cells per well) in DMEM with 10% fetal bovine serum for 24 hours and starved for 48 hours with 1% bovine serum albumin prior to stimulation. ISO (1 µM or 10 µM) was added to the medium. At 24 hours after incubation, the supernatants were aspirated, 10 µl of MTT solution (5 mg/ml) was added to each well, and incubation was continued for 4 hours. After incubation, the supernatants were aspirated and 100 µl of 10% SDS in 10 mM HCl was added. The amount of metabolized MTT was determined with a microplate reader.

Recording of spontaneous activity in myocardial sleeves of pulmonary veins. Pulmonary veins were separated from the atrium at the left atrium–pulmonary vein junction and separated from the lungs at the end of the pulmonary vein myocardial sleeves. Tubular pulmonary veins were cut open and pinned down, endocardial side up, on the bottom of the 20-ml recording chamber. The extracellular solution contained the following: 118.4 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 24.9 mM NaHCO₃, and 11.1 mM glucose, pH 7.4; it was gassed with 95% O₂–5% CO₂ and maintained at 36 ± 0.5°C. Action potentials were recorded in pulmonary vein tissue preparations by using standard microelectrodes inserted from the luminal side. The glass microelectrodes filled with 3M KCl had resistances of 20 to 30 MΩ. The output of a microelectrode amplifier with high input impedance and capacity neutralization (MEZ8201; Nihon Kohden) was digi-

tized by an A/D-converting interface (Power Lab/4SP, AD Instruments) and analyzed using Chart 7 software (AD Instruments) (93, 94).

Statistics. All data are reported as mean ± SEM. Comparison of data was performed using a 2-tailed Student's *t* test when 2 samples were considered or 1-way ANOVA for 3 or more samples. Fisher's exact probability test was used to examine the incidence of spontaneous activity in pulmonary vein myocytes. Differences were considered significant at *P* < 0.05.

Study approval. This study was approved by the Animal Care and Use Committee at Yokohama City University School of Medicine.

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