Exchange Protein Directly Activated by cAMP Mediates Slow Delayed-Rectifier Current Remodeling by Sustained β-Adrenergic Activation in Guinea Pig Hearts

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Rationale: β-Adrenergic receptor activation contributes to sudden death risk in heart failure. Chronic β-adrenergic stimulation, as occurs in patients with heart failure, causes potentially arrhythmogenic reductions in slow delayed-rectifier K⁺ current (Iₖs).

Objective: To assess the molecular mechanisms of Iₖs downregulation caused by chronic β-adrenergic activation, particularly the role of exchange protein directly activated by cAMP (Epac).

Methods and Results: Isolated guinea pig left ventricular cardiomyocytes were incubated in primary culture and exposed to isoproterenol (1 μmol/L) or vehicle for 30 hours. Sustained isoproterenol exposure decreased Iₖs density (whole cell patch clamp) by 58% (P<0.0001), with corresponding decreases in potassium voltage-gated channel subfamily E member 1 (KCNE1) mRNA and membrane protein expression (by 45% and 51%, respectively). Potassium voltage-gated channel, KQT-like subfamily, member 1 (KCNQ1) mRNA expression was unchanged. The β1-adrenergic antagonist 1-[2-((3-Carbamoyl-4-hydroxy)phenoxo)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol dihydrochloride (CGP-20712A) prevented isoproterenol-induced Iₖs downregulation, whereas the β₂-antagonist ICI-118551 had no effect. The selective Epac activator 8-pCPT-2′-O-Me-cAMP decreased Iₖs density to an extent similar to isoproterenol exposure, and adenoviral-mediated knockdown of Epac1 prevented isoproterenol-induced Iₖs/KCNE1 downregulation. In contrast, protein kinase A inhibition with a cell-permeable highly selective peptide blocker did not affect Iₖs downregulation. 1,2-Bis(o-aminophenoxy)ethane-N,N,N',N' -tetraacetate-AM acetoxymethyl ester (BAPTA-AM), cyclosporine, and inhibitor of nuclear factor of activated T cell (NFAT)-calcineurin association-6 (INCA6) prevented Iₖs reduction by isoproterenol and INCA6 suppressed isoproterenol-induced KCNE1 downregulation, consistent with signal-transduction via the Ca²⁺/calcineurin/NFAT pathway. Isoproterenol induced nuclear NFATc3/c4 translocation (immunofluorescence), which was suppressed by Epac1 knockdown. Chronic in vivo administration of isoproterenol to guinea pigs reduced Iₖs density and KCNE1 mRNA and protein expression while inducing cardiac dysfunction and action potential prolongation. Selective in vivo activation of Epac via sp8-pCPT-2′-O-Me-cAMP infusion decreased Iₖs density and KCNE1 mRNA/protein expression.

Conclusions: Prolonged β-adrenergic receptor stimulation suppresses Iₖs by downregulating KCNE1 mRNA and protein via Epac-mediated Ca²⁺/calcineurin/NFAT signaling. These results provide new insights into the molecular basis of K⁺ channel remodeling under sustained adrenergic stimulation. (Circ Res. 2014;114:993-1003.)

Key Words: β-adrenergic receptors ■ arrhythmias, cardiac ■ calcineurin ■ heart failure ■ ion channels

Congestive heart failure (CHF) remains a leading cause of mortality, with arrhythmic sudden death implicated in ≈50% of deaths. Action potential (AP) prolongation is a consistent finding in patients and animal models with CHF. Reduced slow delayed-rectifier potassium K⁺-current (Iₖs) is a particularly common and important finding in CHF-related remodeling. Reduced Iₖs impairs repolarization and promotes arrhythmogenesis, as classically seen with mutations of the underlying subunits potassium voltage-gated channel subfamily E member 1 (KCNE1) and potas-
sium voltage-gated channel, KQT-like subfamily, member 1 (KCNO1) in long-QT syndrome.7

We previously showed that sustained β-adrenergic stimulation decreases $I_{Ks}$ density in the guinea pig,8 but the underlying molecular basis remains poorly understood. β-Adrenergic stimulation causes cardiac remodeling via cAMP, classically mediated by protein kinase A (PKA); however, the novel protein family, exchange protein directly activated by cAMP (Epac), has been shown to mediate β-adrenoceptor actions.9,10 Little is known about the involvement of Epac in cardiac remodeling, particularly at the electrophysiological level. The present study aimed to clarify the molecular mechanisms underlying β-adrenergic downregulation of $I_{Ks}$, with a particular focus on the potential role of Epac.

Methods

For detailed methods description, see the Online Data Supplement.

Cardiomyocyte Isolation

Guinea pigs were injected with heparin (1.0 U/kg) and euthanized by stunning-induced aortic compression following cardiac excision. Hearts were retrogradely perfused with 200 μmol/L Ca$^{2+}$-containing Tyrode solution. When clear, the perfusate was changed to Ca$^{2+}$-free Tyrode solution and digested with 280 U/mg collagenase type II. Cells were obtained by trituration and stored in KRB solution.

Cell Culture and Treatment

Cardiomyocytes were reintroduced to Ca$^{2+}$ by stepwise addition of cell culture medium. Cells were plated and maintained at 37°C in a humidified, 5% CO$_2$-enriched atmosphere. After 2 hours, fresh medium was added and supplemented with 1 μmol/L isoproterenol in drug treatment groups. Cells were kept in culture for an additional 30 hours. In some experiments, 1-[2-(3-carbamoyl-4-hydroxy)phenoxymethyl]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxo]-2-propanol dihydrochloride (CGP-20712A); (±)-1-[2-(3-dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[1-(methylthyl)amino]-2-butanol hydrochloride (ICI-118551); 8-Br-cAMP; 7β-acetoxy-8,13-epoxy-1,6β,9α-trihydroxylad-14-en-11-one (forskolin); 8-pCPT-2′-O-Me-cAMP (8-pCPT); inhibitor of nuclear factor of activated T cell (NFAT)-calcineurin association (NFAT6); cyclosporine; 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetate acetylxyethyl ester (BAPTA)-AM; myristoylated protein kinase A inhibitor peptide (PKI); U-73122; N-[2-N-(4-chlorocinnamyl)-N-methylaminomethyl]phenyl-N-(2-hydroxyethyl)-4-methoxybenzenesulfonamide phosphate salt (KN93); KN92; or N-[4-[2(R)-amino-3-mercaptopropyl]amino-2-(1-naphthalenyl)benzoyl]l-leucine methyl ester trifluoroacetate salt (GGTI) were added to cultured cardiomyocytes along with isoproterenol (1 μmol/L). In other experiments, shRNA in an adenoviral vector, produced based on previously described methods,11,12 was used to knockdown Epac1 (Online Figure I). In all experiments studying effects of blockers on isoproterenol action, cells from the same isolates were exposed in parallel to isoproterenol as an internal control. T-tubule distribution (with di-4-ANEPPS as a marker) and protein-synthesis ([14C]-leucine incorporation) were assessed in isolated cells as described in Methods in the Online Data Supplement.

Electrophysiology

Cell Culture

After 30 hours of exposure to interventions, cardiomyocytes were washed with Tyrode solution before study.

Freshly Isolated Cells After In Vivo Treatment

Cells were studied within 8 hours of isolation. Tight-seal whole-cell patch-clamp technique was used to record currents in voltage-clamp mode. APs were recorded with perforated patch current clamp. All experiments were performed at 36±1°C. For detailed electrophysiological methods, see Methods in the Online Data Supplement. Cell capacitance was 104±4 pF for control and 106±4 pF for isoproterenol-treated cells in culture; 199±11 pF for in vivo vehicle-control and 252±16 pF isoproterenol-treated groups (P<0.05); 146±9 pF for vehicle-control and 180±11 pF for sp-8-pCPT-treated animals (P<0.05) for cells isolated from in vivo-treated animals.

Immunoblots and Immunoochemistry

Membrane protein was denatured and fractionated on 8% SDS-PAGE and then transferred electrophoretically to Immobilon-P polyvinylidene fluoride membranes. Membranes were incubated with primary antibodies overnight and then exposed to secondary antibodies. All results were normalized to GAPDH immunoblots. Immunohistochemistry was used to quantify membrane expression of KCNE1 protein and nuclear translocation of NFAT.

Real-Time Polymerase Chain Reaction

For RNA isolation and quantitative PCR methods, see Methods in the Online Data Supplement. Gene expression levels were normalized to the geometric average of multiple reference genes.13

In Vivo Models

Guinea pigs received daily intraperitoneal injections of isoproterenol or vehicle. Isoproterenol was injected at an initial dose of 50 μg/kg per day. The dose was increased 100 μg/kg per day every week for 13 weeks. To produce in vivo Epac activation, sp-8-pCPT was administered via osmotic minipump (16 μg/d) for 6 weeks; vehicle-filled minipumps were used for parallel control animals. Echocardiography was used to assess cardiac function changes14 in isoproterenol-treated and parallel control animals, as detailed in Methods in the Online Data Supplement.
Data Analysis
Clampfit 9.0 (Axon) and GraphPad Prism 5.0 were used for data analysis. Group comparisons were performed with unpaired Student t tests (for single 2-group comparisons) or 1-way ANOVA with Bonferroni-corrected t tests (for multiple-group comparisons). Data are expressed as mean±SEM.

Results
Sustained β-Adrenergic Stimulation Decreases IKs
We first established the stability of the guinea pig cell culture system in vitro (Online Figure II). IKs density, as well as protein expression of the underlying KCNQ1 and KCNE1 subunits, was stable in the absence of isoproterenol. Isoproterenol treatment increased cell area by ≈50% (Online Figure IIIA), did not affect cell capacitance (Online Figure IIIIB), and increased leucine incorporation (Online Figure IIIIC). T-tubule density decreased in culture, with significantly greater decreases in isoproterenol-treated cells versus parallel controls (Online Figure IIIID and IIIE), potentially accounting for unchanged capacitance in isoproterenol-treated cells, despite increased cell size.

IKs recordings from control and isoproterenol (1 μmol/L)-treated cells are shown in Figure 1A and 1B. Figure 1C and 1D shows overall current density/voltage relations, indicating a significant decrease (by ≈60%) in isoproterenol-treated cells. Current densities normalized to maximum

Figure 1. Effects of isoproterenol (Iso) on slow delayed-rectifier potassium current (IKs) and action potentials (APs). A to D, in vitro effects. A and B, Original recordings in cells incubated with control and isoproterenol containing medium, respectively. Voltage protocol (4-s depolarizing pulses at 0.1 Hz, followed by 3 seconds at −30 mV to observe tail currents). C, IKstep density–voltage relations for cells cultured in the presence of Iso or vehicle (control [CTL]). D, Tail current (IKTAIL) density–voltage relations. E to H, Effects of chronic in vivo isoproterenol administration on IKs. E and F, Original recordings in cells incubated with control and isoproterenol containing medium, respectively. G, IKstep density–voltage relations in freshly isolated ventricular cardiomyocytes from isoproterenol-treated and CTL animals. H, Tail current (IKTAIL) density–voltage relations.

Figure 2. Relationship between isoproterenol (Iso) effects and β-receptor subtype, cAMP and protein kinase A inhibitor (PKI). Left. Slow delayed-rectifier potassium current (IKs) recordings (+50 mV) and right, mean±SEM data; under vehicle-culture (control [CTL]) and (A and B) Iso, β1-(1-[2-(3-carbamoyl-4-hydroxy)phenoxy]ethylamino)-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol dihydrochloride [CGP-201712A]) and β2-((±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[1-methylethyl]amino)-2-butanol hydrochloride [ICI-185516]) adrenoceptor antagonists (***P<0.001, Iso vs CTL; ###P<0.001, Iso+β1-blocker vs Iso-alone); (C and D) 8-bromo-cAMP (8-Br-cAMP) (***P<0.001, Iso vs CTL; ###P<0.001, 8-Br-cAMP vs CTL); (E and F) 7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxyabd-14-en-11-one (forskolin), 8-Br-cAMP+PKI (***P<0.001, forskolin vs CTL; ###P<0.001, 8-Br-cAMP+PKI vs CTL) and PKI (***P<0.001, Iso vs CTL; ###P<0.001, PKI+Iso vs CTL). Voltage protocol at top right. Treatment duration for all drugs including isoproterenol was 30 hours. n indicates number of cells; and TP, test potential.
values in each cell (Online Figure IVA and IVB) superimposed, indicating that isoproterenol treatment did not affect voltage dependence. Half-activation voltages (Boltzmann fit) averaged +34.6±1.3 and +33.2±2.5 mV in control (n=13) and isoproterenol-treated (n=8) cells, respectively (P=NS). Isoproterenol exposure accelerated \( I_{\text{Ks}} \) activation by reducing the slow-phase time constant (Online Figure IVC and IVD).

**Involvement of \( \beta_1 \)-Adrenoceptors and cAMP Signaling**

Cells incubated with isoproterenol and highly selective \( \beta_1 \) (CGP-20712A) or \( \beta_2 \) (ICI-118551) antagonists were compared with parallel control and isoproterenol-alone groups. Figure 2A shows representative \( I_{\text{Ks}} \) recordings. Corresponding current density/voltage relationships (Figure 2B) indicate that concomitant treatment with the \( \beta_1 \)-blocker CGP-20712A abolished the isoproterenol effect. The \( \beta_2 \)-blocker ICI-118551 failed to alter isoproterenol action, confirming that the isoproterenol effect is mediated through \( \beta_1 \)-adrenergic receptors.

After \( \beta_1 \)-adrenoceptor activation, the trimeric G-protein complex releases \( G_{\alpha_i} \), which activates adenylyl cyclase, increasing intracellular cAMP levels.\(^{15}\) Sustained exposure to the cell-permeable cAMP agonist 8-bromo-cAMP reduced \( I_{\text{Ks}} \) (Figure 2C), mimicking isoproterenol effects (Figure 2D). Similar changes were observed with forskolin, which increases intracellular cAMP levels by directly activating adenylyl cyclase (Figure 2E and 2F). Acute \( I_{\text{Ks}} \) enhancement caused by \( \beta_1 \)-adrenergic stimulation is mediated by PKA activation/phosphorylation of KCNQ1 on Ser-27.\(^{16}\) To evaluate the role of PKA in \( I_{\text{Ks}} \) downregulation, cardiomyocytes were exposed for 30 hours to isoproterenol in the presence of the N-myristoylated (cell permeable) form of the peptide PKA-inhibitor PKI (1 \( \mu \)mol/L). PKI did not suppress isoproterenol-induced \( I_{\text{Ks}} \) downregulation (Figure 2G and 2H). In contrast, PKI blunted \( I_{\text{Ks}} \) enhancement resulting from acute isoproterenol exposure (Online Figure VA and VB), indicating that the persistent chronic-isoproterenol effect in the presence of PKI is not because of inactivity of PKI. In addition, chronic treatment of cells with 8-Br-cAMP plus PKI suppressed \( I_{\text{Ks}} \), further excluding the involvement of PKA (Figure 2E and 2F).

**Involvement of Epac**

To assess the involvement of Epac, we treated cardiomyocytes with 6-\( \mu \)mol/L 8-pCPT-2’-O-Me-cAMP (8-pCPT), a highly selective Epac activator.\(^{17}\) Sustained Epac activation with 8-pCPT reduced \( I_{\text{Ks}} \) densities to values comparable with those in a

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**Figure 3. Effects of isoproterenol (Iso), exchange protein directly activated by cAMP (Epac) activation and Epac knockdown. A to E. In vitro studies.** A, Slow delayed-rectifier potassium current \( (I_{\text{K}}) \) recordings at +50 mV under vehicle-control (CTL), Iso, and 8-pCPT-2’-O-Me-cAMP. B, \( \text{Mean±SEM } I_{\text{Ks}} \) density–voltage relations. C, \( I_{\text{Ks}} \) recordings for cells infected with scrambled construct (Scr+Iso)-virus, or Epac knockdown probe (Epac1KD+Iso), and cells cultured in the presence of Iso. D, \( \text{Mean±SEM } I_{\text{Ks}} \) density–voltage relations **P<0.01, ***P<0.001 vs CTL; ##P<0.01, ###P<0.001 vs Iso-alone. E, \( \text{Mean±SEM } I_{\text{Ks}} \) densities at +60 mV. **P<0.01, ***P<0.001 for comparison shown. F to H. Effects of in vivo sp-8-pCPT administration. F, \( I_{\text{Ks}} \) recordings from animals treated with sp-8-pCPT and vehicle (CTL). G, \( \text{Mean±SEM tail current } (I_{\text{Ks, tail}}) \) density–voltage relations. H, Representative action potential (AP) recordings (1 Hz) from sp-8-pCPT-treated animals and CTL (left); AP duration (APD) at 50% (APD50) and 90% (APD90) repolarization (right). n indicates number of cells; and TP, test potential.
parallel isoproterenol-treated group (Figure 3A; eg, at +50 mV, from 3.3±0.4 pA/pF in control to 1.7±0.2 pA/pF in isoproterenol and 1.4±0.2 pA/pF in sp-8-pCPT).

Figure 3A and 3B shows that Epac stimulation can mimic the effect of isoproterenol, but to establish the role of Epac as a mediator of isoproterenol-induced \( I_{Ks} \) downregulation, it is necessary to assess the effects of Epac inhibition on isoproterenol action. In the absence of a specific pharmacological inhibitor, we turned to genetic knockdown. Two isoforms of Epac (Epac1 and Epac2) are encoded by distinct genes (RAPGEF3 and RAPGEF4).18 Epac1 is highly expressed in the heart, kidneys, ovaries, and thyroid glands, whereas Epac2 is predominant in the brain and pituitary.19 Furthermore, isoproterenol treatment enhanced the expression of Epac1 in our in vitro system (Online Figure VIA) and not that of Epac2 (Online Figure VIB) and increased the Epac1/2 expression ratio (Online Figure VIC). Based on these data, we decided to target Epac1 and designed a specific shRNA (Online Figure I), along with a scrambled control sequence, each inserted in bicistronic adenoviral delivery vectors incorporating green fluorescent protein. Incubation with the Epac1 knockdown-virus attenuated Epac1 expression after isoproterenol exposure (Online Figure VIIA), whereas Epac2 expression was unaffected (Online Figure VIIIB). The scrambled-virus did not alter Epac expression in the presence of isoproterenol (Online Figure VIIIA), and isoproterenol significantly increased Epac1 expression in the presence of scrambled-virus versus scrambled-virus incubation alone (Online Figure VIIIB). Figure 3C shows representative \( I_{Ks} \) recordings in cells treated with isoproterenol in the presence of the scrambled-control virus, knockdown-virus, and virus noninfected control, respectively. Epac1 knockdown suppressed isoproterenol-induced downregulation of \( I_{Ks} \), as compared with isoproterenol-alone and scrambled sequence (Figure 3D and 3E). These data are strong evidence for a central role of Epac1 in isoproterenol-induced \( I_{Ks} \) downregulation.

Role of Ca\(^{2+}\)/Calcineurin/NFAT

Epac action is commonly transduced by increased intracellular Ca\(^{2+}\) levels.20 To determine the role of cell Ca\(^{2+}\) in mediating effects of Epac in our system, we used a cell-permeable calcium chelator (BAPTA-AM, 10-μmol/L). Cardiomyocytes incubated with isoproterenol and BAPTA-AM did not show a reduction in \( I_{Ks} \) current density on isoproterenol exposure (Figure 4A), whereas cells from the same isolates exposed to isoproterenol showed typical \( I_{Ks} \) suppression.

We then turned our attention to potential downstream Ca\(^{2+}\)-dependent mediators of Epac action. Calcineurin is a Ca\(^{2+}\)-activated phosphatase that is known to mediate Epac-induced cardiac hypertrophy.10 To assess the role of calcineurin, cardiomyocytes were treated with the calcineurin blocker cyclosporine A (0.8 μmol/L). Cyclosporine prevented the isoproterenol-induced downregulation of \( I_{Ks} \) (Figure 4B). A major mediator of calcineurin action is the nuclear factor of activated T-lymphocytes, which is dephosphorylated by calcineurin, allowing increased transport into the nucleus and enhanced transcription factor action.11 Figure 5A shows enhanced nuclear localization of NFATc4 (red) and NFATc3 (green) following isoproterenol exposure. Overall nuclear localization was increased for both NFATc3 (by ≈61%; \( P<0.01 \)) and NFATc4 (≈42%; \( P<0.05 \)) by isoproterenol incubation (Figure 5B). To assess the functional role of NFAT in \( I_{Ks} \) downregulation, we treated cardiomyocytes with a cell-permeable NFAT blocker (inhibitor of NFAT-calcineurin association-6; 1-μmol/L), which prevented \( I_{Ks} \) downregulation by isoproterenol (Figure 5C and 5D). Epac1 knockdown suppressed β-adrenergically mediated translocation of NFATc3 and c4 into the nucleus, confirming NFAT downregulation as a mechanism downstream to isoproterenol-induced Epac activation (Online Figure IX).

In Vivo Models

Chronic in vivo β-adrenergic stimulation increased left ventricular mass/body weight ratio, indicating cardiac hypertrophy (Online Table II). Echocardiography showed significant impairments in left ventricular ejection fraction and fractional shortening (Online Table III). Figure 1E and 1F shows representative \( I_{Ks} \) recordings from control and isoproterenol-treated animals. \( I_{Ks} \) density was significantly reduced, by ≈65% (Figure 1G and 1H). In vivo isoproterenol administration did not alter \( I_{Ks} \) voltage dependence (Online Figure IVE and IVF) but significantly accelerated \( I_{Ks} \) activation (Online Figure IVG and IVH), similar to the effect observed in vitro. Of note, in vivo isoproterenol administration did not cause detubulation (Online Figure IIIF and IIII). AP duration was significantly increased in isoproterenol-treated animals (Figure 1I and 1J). The expression of both Epac1 and Epac2 mRNA was increased (Online Figure VIF and VIE), but the increase in Epac2 was larger than in Epac1, decreasing the Epac1/Epac2 expression ratio (Online Figure VIIF). In vivo isoproterenol also remodelled other ionic currents, reducing L-type calcium current (\( I_{CaL} \)) density by ≈45% and inward rectifier current (\( I_{K1} \)) by ≈77% (Online Figure X). In vivo administration of the Epac activator sp-8-pCPT decreased \( I_{Ks} \) by ≈64%, reproducing the effect of isoproterenol (Figure 3F and 3G). AP duration was significantly prolonged in sp-8-pCPT–treated animals (Figure 3H). As was the case for isoproterenol, \( I_{CaL} \) and \( I_{K1} \) were reduced (by ≈30% each) in sp-8-pCPT–treated animals (Online Figure XI).

Figure 4. Effects of intracellular Ca\(^{2+}\) buffering and calcineurin inhibition. A, Left, Slow delayed-rectifier K+ current \( (I_{K}) \) recordings at +50 mV after culture in vehicle-control (CTL), isoproterenol (Iso) or 1,2-bis(o-aminophenoxy)ethane-N,N,N,N′-tetraacetate acetoxymethyl ester (BAPTA)-AM plus Iso (BAPTA-AM+Iso). Right, Corresponding mean±SEM \( I_{K} \) density–voltage relations. B, \( I_{Ks} \) recordings at +50 mV after culture in CTL, Iso, and cyclosporine A plus Iso (Cyclo+Iso) media. Right, Corresponding mean±SEM \( I_{Ks} \) density–voltage relationships. *\( P<0.05 \), **\( P<0.001 \) vs CTL. n indicates number of cells; and TP, test potential.
To further address the mechanisms underlying $I_{Ks}$ downregulation, we assessed mRNA expression for the $I_{Ks}$ subunits KCNQ1 and KCNE1. KCNQ1 mRNA expression was not significantly altered (Figure 5E), but KCNE1 mRNA expression was clearly reduced, by $\approx 45\%$ (Figures 5F and 6A). These results suggest KCNE1 as the downstream target of the Epac1-stimulated Ca$^{2+}$/calcineurin/NFAT system. Numerous NFAT-binding sites are located on the 5’-upstream region of the guinea pig transcriptional start site for KCNE1, including 1 within 300 bp (Online Figure XII). The NFAT blocker inhibitor of NFAT-calcineurin association-6 (INCA6) suppressed KCNE1 downregulation (Figure 5F), without altering KCNQ1 expression (Figure 5E), consistent with NFAT-mediated regulation. Representative KCNE1 immunoblots are shown, along with mean data, in Figure 6A. Isoproterenol incubation reduced KCNE1 protein expression significantly, by $56\%$. The protein expression changes were further confirmed via immunostaining (Figure 6B), which showed reduced membrane expression of KCNE1 protein (by $\approx 82\%$; $P<0.01$) in response to sustained in vitro isoproterenol exposure. Corresponding in vivo results are shown in Figure 6C and 6D. KCNE1 protein and mRNA expression were significantly decreased after in vivo isoproterenol administration (Figure 6C). Similar changes were seen with in vivo sp-8-pCPT infusion (Figure 6D).

Molecular Basis of Isoproterenol/Epac Effect on $I_{Ks}$

To further address the mechanisms underlying $I_{Ks}$ downregulation, we assessed mRNA expression for the $I_{Ks}$ subunits KCNQ1 and KCNE1. KCNQ1 mRNA expression was not significantly altered (Figure 5E), but KCNE1 mRNA expression was clearly reduced, by $\approx 45\%$ (Figures 5F and 6A). These results suggest KCNE1 as the downstream target of the Epac1-stimulated Ca$^{2+}$/calcineurin/NFAT system. Numerous NFAT-binding sites are located on the 5’-upstream region of the guinea pig transcriptional start site for KCNE1, including 1 within 300 bp (Online Figure XII). The NFAT blocker inhibitor of NFAT-calcineurin association-6 (INCA6) suppressed KCNE1 downregulation (Figure 5F), without altering KCNQ1 expression (Figure 5E), consistent with NFAT-mediated regulation. Representative KCNE1 immunoblots are shown, along with mean data, in Figure 6A. Isoproterenol incubation reduced KCNE1 protein expression significantly, by $56\%$. The protein expression changes were further confirmed via immunostaining (Figure 6B), which showed reduced membrane expression of KCNE1 protein (by $\approx 82\%$; $P<0.01$) in response to sustained in vitro isoproterenol exposure. Corresponding in vivo results are shown in Figure 6C and 6D. KCNE1 protein and mRNA expression were significantly decreased after in vivo isoproterenol administration (Figure 6C). Similar changes were seen with in vivo sp-8-pCPT infusion (Figure 6D).

The results above indicated an important role for Ca$^{2+}$/calcineurin/NFAT signaling but do not exclude the involvement of other molecular pathways. Ras-related protein 1 (Rap1) is known to be activated after Epac activation.$^{18}$ Its potential role was assessed by incubating cells with GGTI (a Rap1 blocker). GGTI prevented isoproterenol-induced $I_{Ks}$ reduction (Online Figure XIIIA and XIIIB). Phospholipase C (PLC) is another downstream effector of some Epac1 effects.$^{22}$ Concomitant treatment of cells with isoproterenol and U-73122 (a phospholipase C inhibitor) did not prevent isoproterenol-induced reductions in $I_{Ks}$ density (Online Figure XIIIC and XIIID). Ca$^{2+}$/calmodulin-dependent kinase type II (CaMKII) is known to be activated by $\beta_1$-adrenergic stimulation.$^{23}$ Concomitant stimulation of cells with isoproterenol and KN93 (a CaMKII blocker) prevented reductions in $I_{Ks}$ density, whereas the inactive congener KN92 was ineffective (Online Figure XIIIIE and XIIIIF), indicating the necessity for intact CaMKII activity for the isoproterenol effect. In the absence of adrenergic stimulation, neither GGTI nor KN93 altered $I_{Ks}$ (Online Figure XIV).

**Discussion**

In this study, we found that chronic $\beta$-adrenergic stimulation decreases $I_{Ks}$ density both in vitro and in vivo while...
downregulating KCNE1 subunits. Detailed characterization in vitro showed that this effect is mediated via Epac signaling through the Ca\(^{2+}\)/calcineurin/NFAT pathway. A summary of our experimental observations and the mechanistic model they suggest is provided in Figure 7.

**Remodeling of Delayed-Rectifier K\(^+\) Currents**

The delayed-rectifier K\(^+\) current system is crucial for cardiac repolarization in mammals. \(I_{Kn}\) downregulation occurs in patients with terminal CHF\(^3\) and in ventricular and atrial cells from different animal models.\(^{3,24–30}\) Animal models of hypertrophy also show reduced \(I_{Kn}\).\(^3\) Atrioventricular block–induced remodeling also decreases \(I_{Kn}\) in ventricular cardiomyocytes.\(^{32,33}\) Less is known about the signal-transduction mechanisms that lead to \(I_{Kn}\) downregulation and the underlying changes in \(I_{Kn}\) subunits. Prior studies have provided discrepant results. Tsuji et al\(^{24}\) showed a decrease in both KCNQ1 and KCNE1 subunits in rabbits with tachypacing-induced heart failure, with a corresponding change in the protein. However, other studies of tachypacing-induced CHF in dogs\(^6\) and rabbits\(^3\) did not show changes in KCNQ1 and KCNE1 mRNA or protein expression. Borlak et al\(^{34}\) reported an increase in KCNQ1 and KCNE1 subunit mRNA in heart samples from humans with end-stage CHF. Some of the discrepancies may be because of differences in the severity and duration of CHF, as well as species and drug therapy conditions. The QT-interval prolongation associated with K\(^+\)-channel downregulation is a significant predictor of sudden cardiac death in patients with CHF.\(^3\)

**Epac Signaling in Cardiac Remodeling**

cAMP, the universal second messenger that is produced via adenylyl cyclase after \(\beta\)-receptor activation, plays an important role in cardiovascular physiology. Although PKA is the primary effector of cAMP, other more recently identified proteins, such as Epac, represent important signaling mechanisms downstream to cAMP. Here, we report that chronic in vitro stimulation of \(\beta_1\)-adrenergic receptors activates Epac1, which decreases \(I_{Kn}\) density independently of PKA. In vivo, isoproterenol administration increases Epac expression, and its effects are mimicked by an Epac agonist. Epac1 (RAPGEF3) mRNA is highly expressed in heart.\(^3\) Myocardial Epac1 expression increases in rats with pressure-overload induced by aortic constriction and in rat

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**Figure 6. Potassium voltage-gated channel subfamily E member 1 (KCNE1) expression changes.** A and B. In vitro studies. A, Top, Crude membrane protein extracts and RNA extracts were obtained from cells cultured in control-vehicle (CTL) and isoproterenol-medium (Iso). KCNE1 bands were seen on immunoblot at the expected molecular mass of \(\approx20\) kDa. Bottom, Mean±SEM expression levels for KCNE1 protein relative to GAPDH bands on the same lanes (\(*P<0.05\), and mRNA expression (\(**P<0.001\)). B, Immunostaining for KCNE1 and mean±SEM membrane fluorescence intensity. C and D, In vivo model results. C, Top, Immunoblots for membrane KCNE1 protein in cardiomyocytes from animals treated with isoproterenol or vehicle (CTL). Bottom, Left, Means±SEM protein expression levels (\(*P<0.05\); right, mRNA expression. D, Top, Immunoblots for membrane KCNE1 protein in animals treated with sp-8-pCPT or vehicle. Bottom, Left, Means±SEM protein expression levels; right, mRNA expression (\(*P<0.05, \***P<0.001\)). n indicates numbers of independent experiments, each from 1 heart.
ventricular cardiomyocytes treated with isoproterenol. Epac1 and Epac2 are also upregulated in the hearts of mice subjected to chronic-isoproterenol infusion. Epac1 expression is increased 2-fold in ventricular cardiomyocytes from patients with CHF, with no change in Epac2 expression. Thus, cardiac Epac expression increases under cardiac-load and adrenergic-stimulation conditions that cause hypertrophy and remodeling. There is extensive evidence for a causative role of Epac in cardiac hypertrophy. Little is known about the role of Epac in cardiac electrophysiology. Epac activation inhibits ATP-sensitive K⁺-channels in pancreatic β-cells and Epac1 coimmunoprecipitates with SUR1, a subunit of the Kᵦᵦᵦ-channel. Exposure of rat chromaffin cells to 8-pCPT increases T-type Ca²⁺-current and Ca₃.1-subunit expression. Acute perfusion of rat and mouse cardiomyocytes with 8-pCPT does not affect L-type Ca²⁺ current, but the Epac activator 8-4-(chlorophenylthio)-2'-methyladenosine-3',5' monophosphate (8-pCPT) strongly enhances Ca²⁺-induced Ca²⁺ release in mouse cardiomyocytes. Acute Epac activation failed to induce any changes in AP duration in 2 studies; however, a more recent investigation showed AP duration increases in rats after acute 8-pCPT-acetoxymethyl-ester perfusion.

Epac activation increases Ca²⁺ sparks via CaMKII phosphorylation of ryanodine receptors in rat cardiomyocytes. A recent elegant study showed that in vivo infusion of an Epac activator to rats elicits a PKA-independent positive inotropic response, increases cardiomyocyte Ca²⁺ transients, enhances sarcoplasmic reticulum Ca²⁺ stores and Ca²⁺ transients, and promotes Ca²⁺-dependent arrhythmic activity. Inhibition of calcineurin or CaMKII prevented Epac-induced Ca²⁺ responses.

The present study is the first to implicate Epac in Iₖᵦᵦᵦ downregulation. The Epac dependence of adrenergically induced Iₖᵦᵦᵦ downregulation was established by the ability of direct Epac activation to mimic adrenergic effects, the lack of change with PKA inhibition, and the suppression of adrenergic effects on Iₖᵦᵦᵦ and KCNE1 expression when Epac was knocked down. Signaling was Ca²⁺ dependent (as evidenced by the effect of BAPTA) and required intact calcineurin action (shown by suppression with cyclosporine A). NFAT translocation was a central event: blockade of calcineurin-induced NFAT dephosphorylation with INCA6 prevented Iₖᵦᵦᵦ and KCNE1 downregulation, and the suppression of isoproterenol-induced Iₖᵦᵦᵦ downregulation by Epac knockdown was accompanied by the prevention of NFAT translocation to the nucleus. The signaling system that we uncovered is consistent with prior studies of Epac effects in the heart. Calcineurin activity is increased in cells treated with 8-pCPT, and Epac activation is known to significantly increase NFAT nuclear translocation, which is important for the induction of cardiac hypertrophy.

**Figure 7. Schematic representation of the mechanisms involved in slow delayed-rectifier K⁺ current (Iₖᵦᵦᵦ) downregulation by sustained β-adrenergic stimulation.** Blockers (red) and activators (blue) were used to probe specific components of the pathway. 8-pCPT indicates 8-pCPT-2'-O-Me-cAMP; AC, adenyl cyclase; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetate acetoxymethyl ester; CaMKII, Ca2+/calmodulin-dependent protein kinase type II; CGP-20712A, 1-[2-((3-carboxamidoyl)-4-hydroxy)phenoxy]ethanolamin-o]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol dihydrochloride; CN, calcineurin; Epac, exchange protein directly activated by cAMP; INCA6, inhibitor of NFAT-calcineurin Association-6; KCNE1, potassium voltage-gated channel subfamily E member 1; KCNQ1, potassium voltage-gated channel, KQT-like subfamily, member 1; NFAT, nuclear factor of activated T cell; PKI, protein kinase A inhibitor peptide; Rap1, Ras-related protein 1. 

**Relationship to Other Signaling Systems in K⁺ Channel Remodeling**

Rossow et al have shown spatial heterogeneity of NFATc3-dependent Iₖᵦᵦᵦ downregulation, causing a loss of the normal transmural gradient in mouse ventricular cardiomyocytes after chronic in vivo isoproterenol infusion. β-Adrenergic stimulation increased intracellular Ca²⁺, calcineurin, and NFAT activity, which reduced Kv4.2 expression and Iᵦᵦᵦ density. The upstream pathway was not identified. In mice with myocardial infarction, downregulation of Iᵦᵦᵦ and Iᵦᵦᵦ₁₂₃ was prevented by calcineurin inhibition or NFATc3 knockout. Although calcineurin/NFAT signaling suppresses Iᵦᵦᵦ₁₂₃ transcription in most studies, Iᵦᵦᵦ₁₂₃ upregulation occurs in neonatal rat cardiomyocytes. Although Cav1.2 is downregulated via the same pathway in canine cardiomyocytes, NFAT is an important downstream mediator of responses to changes in intracellular Ca²⁺, our data are the first showing a role in downregulating delayed-rectifier K⁺ currents.

We also identified the involvement of other signaling molecules, such as CaMKII and Rap1, in Iᵦᵦᵦ₁₂₃ regulation. Previous studies have identified a role for CaMKII in Iᵦᵦᵦ₁₂₃ downregulation in tachycardia remodeling of canine ventricular cardiomyocytes. Interestingly, in that work, the primary signaling system involved was the Ca²⁺/calcineurin/NFAT system, but intact CaMKII function was necessary for K⁺ current downregulation. CaMKII activation also suppresses
delayed-rectifier K+ currents in neurons. Transgenic CaMKIIδc overexpression reduces Kir2.1 expression and I\textsubscript{Ks} in mice. CaMKII-expression is increased in calcineurin-transgenic mice; CaMKII-inhibitory drugs improve left ventricular function and prevent arrhythmias. Less is known about the role of Rap1 in cardiac electrophysiology. Rap1, along with phospholipase C, participates in Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}-release after β-adrenergic stimulation and Epac activation. It is possible that CaMKII and Rap1 contribute to Ca\textsuperscript{2+} liberation, which we found was essential for I\textsubscript{Ks} downregulation. Prior studies have demonstrated a role for Rap1 and CaMKII in Epac-induced increases of murine Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release, although intact phospholipase C was also needed. Additional work will be needed to clarify the detailed molecular signaling associated with these molecules.

**Novel Findings and Potential Significance**

Our study is the first to define the mechanisms underlying I\textsubscript{Ks} downregulation induced by chronic β-adrenergic stimulation. It is also the first to show a central role of Epac signaling in the control of K\textsuperscript{+} channel expression. Our findings may be relevant to the prevention of malignant arrhythmias in a variety of contexts. Sympathoadrenergic activation is an important contributor to arrhythmic risk in patients with CHF, as well as in animal models. It may become possible to target Epac-mediated electric remodeling to prevent potentially lethal arrhythmic events. β-Adrenoceptor blockers are the mainstay of therapy to prevent arrhythmic events in long-QT syndrome patients. Their protective action is reasonably attributed to the suppression of acute electrophysiological effects of adrenergic stimulation; however, they may also act to maintain repolarization reserve that might otherwise be suppressed by downregulation of I\textsubscript{Ks} through chronically elevated background adrenergic tone. The Epac system was described relatively recently, and our knowledge about its role in cardiac pathophysiology is rather limited. Our study is the first to implicate Epac in cardiac ion-channel remodeling and to detail the associated signaling pathway. More work is needed to establish the role of Epac signaling in other aspects of cardiac electric remodeling.

One potentially interesting and novel aspect of the remodeling we observed was a change in the kinetics of I\textsubscript{Ks} with chronic exposure to isoproterenol or agents that mimicked its signaling like 8-bromo-cAMP and 8-pCPT. The KCNE1 subunit is known to contribute importantly to the formation of I\textsubscript{Ks} channels, slowing activation and enhancing current density. The kinetic changes that we observed may therefore be caused by selective downregulation of KCNE1, with consequent changes in KCNE1:KCNQ1 stoichiometry.

**Potential Limitations**

We used an in vitro primary culture system of adult ventricular cardiomyocytes, with an animal system that, unlike mouse and rat models, has important delayed-rectifier K\textsuperscript{+} currents of the type important for human cardiac repolarization. The use of this in vitro system allowed the exploration of detailed mechanisms with probes not readily applicable in vivo. Changes in cardiomyocyte properties over time in culture are a potential problem, but we established the stability of I\textsubscript{Ks} density and associated subunits in culture. In addition, the density of I\textsubscript{Ks} sometimes varied among different sets of cells. We therefore included internal controls (generally, cells cultured in vehicle and isoproterenol) for each set of experiments. Thus, each data set shown consists of simultaneously cultured/studied cells from each isolate. There are important differences in I\textsubscript{Ks} properties among species. Caution is therefore needed in extrapolating our results to other species, especially humans.

We observed cellular hypertrophy after chronic-isoproterenol exposure in terms of increased cell dimensions, but not capacitance. The discrepancy is likely related to the detubulation that occurs in cultured cardiomyocytes, which was exaggerated by isoproterenol and reduces the effective cell membrane surface area (Online Figure III). Chronic in vivo isoproterenol stimulation produced similar changes in I\textsubscript{Ks} and KCNE1 expression to those seen with in vitro treatment, despite no evidence of detubulation and a significant increase in cell capacitance. Interestingly, in vivo Epac administration reproduced the I\textsubscript{Ks} remodeling effects of isoproterenol.

The electrophysiological consequences of background adrenergic tone in vivo will reflect the chronic ion-channel remodeling effects plus any additional changes because of ongoing (acute) adrenergic signaling. The ion-channel remodeling we observed affected adrenergically enhanced outward K\textsuperscript{+} current (60% decrease in I\textsubscript{Ks} more than inward Ca\textsuperscript{2+} current (45% reduction). Thus, any acute adrenergic effects would be expected to increase inward current more than outward and to further delay repolarization. Additional work is clearly needed to define the mechanisms of adrenergic regulation of ion channels other than I\textsubscript{Ks}, to determine the systems effects of chronically elevated adrenergic tone in vivo and to assess their specific role in disease-state paradigms like CHF.

We performed in vivo experiments to determine whether the phenomena we observed under cell-culture conditions in vitro also pertain to the effects of sustained β-adrenergic stimulation in vivo. We based our in vivo study conditions for isoproterenol on previous studies in the guinea pig, which showed that significant changes in cardiac structure/function/electrophysiology required 3 months of incremental intraperitoneal therapy. We based the Epac regimen on prior studies in rats, which used continuous infusion for 4 weeks, but we increased the duration of therapy to 6 weeks, the maximum duration possible with our osmotic minipumps, because of anticipated potential species differences. In view of differences in exposure period, dose, etc, the different series we performed can only be compared qualitatively: in vivo isoproterenol and sp-8-pCPT produced similar effects to each other, with changes consistent with our in vitro observations. The in vitro model allowed us to perform extensive detailed mechanistic studies that could not be practically executed in vivo, whereas the in vivo studies allowed us to confirm that the phenomena we observed in vitro are applicable to in vivo conditions. We used sp-8-pCPT as an Epac-selective agonist, as have many prior studies, but sp-8-pCPT products can have effects on other signaling systems. We confirmed the role of Epac signaling in vitro with adenosinergic-mediated knockdown; however, we were unable to apply gene knockdown in vivo; this limitation should be considered in interpreting our results.

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Disclosures

None.

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Exchange Protein Directly Activated by cAMP Mediates Slow Delayed-Rectifier Current Remodeling by Sustained β-Adrenergic Activation in Guinea Pig Hearts

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Supplemental Material

Exchange Protein Directly Activated by cAMP (Epac) Mediates Slow Delayed-Rectifier Current Remodeling by Sustained Beta-Adrenergic Activation in Guinea Pig Hearts

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Supplemental Materials and Methods

Guinea Pig Ventricular-cardiomyocyte Isolation
Animal care and handling procedures complied with the guidelines established in the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the ethics committee of the Montreal Heart Institute. Guinea pigs weighing 350-450 g were injected with heparin (1.0 units/kg) and euthanized by stunning-induced coma with loss of all reflex-responses, followed by cardiac excision. The heart was quickly excised and transferred to ice-cold oxygenated Tyrode solution (mmol/L: NaCl 136, KCl 5.4, MgCl2 1, HEPES, Na2H2PO4 0.33; pH adjusted to 7.35). The heart was retrogradely-perfused via the aorta with 200-µmol/L Ca2+-containing Tyrode solution. When clear, the perfusate was changed to Ca2+-free Tyrode solution and digested with the addition of 280- U/mg collagenase type-II (Worthington; 25 mg in 50 mL Tyrode solution) and 1% bovine serum albumin (Bioshop). Cells were obtained by trituration and stored in KB solution containing (mmol/L): KCl 20, KH2PO4 10, glucose 10, mannitol 40, albumin 0.1%, L-glutamic acid 70, β-hydroxybutyric 10, taurine 20, EGTA 10; (pH 7.35). Cells were concentrated and allowed to settle by gravity. The pellet was kept for cell culture.

Cell Culture and Drug Treatment
Cardiomyocytes were reintroduced to calcium by a stepwise addition of cell-culture medium (Hyclone M199+Earle’s salts and L-glutamine) to the cells resuspended in KB (successively 200, 500, 1000, and 1800 µmol/L). The medium was supplemented with Na-penicilllin and streptomycin (Hyclone, 1-µg/mL) and insulin-transferrin-selenium-X (GIBCO-1%). Cells were plated at low density (~10^4 cells/cm²) on glass coverslips and culture dishes coated with laminin (Sigma, 20-µg/mL) and maintained at 37°C in a humidified, 5% CO2-enriched atmosphere. After 2 hours, dead cells were removed. Fresh medium was added and supplemented with 1-µmol/L isoproterenol (Iso, Sigma) in drug-treatment groups. Cells were kept in culture for an additional 30 hours. In some experiments, CGP-20712A (Sigma, 300-nmol/L), ICI-118551 (Sigma, 500-nmol/L), 8-Br-cAMP (Sigma, 1-mmol/L), forskolin (Calbiochem, 10-µmol/L), 8-pCPT (Sigma, 6-µmol/L), INCA-6 (Calbiochem, 1- µmol/L), cyclosporine (Sigma, 0.8-µmol/L), BAPTA-AM (Santa Cruz, 10-µmol/L), myristoylated (cell-permeable) PKI (Invitrogen, 1-µmol/L), U-73122 hydrate (Sigma, 1-µmol/L), KN93 (Calbiochem, 500-nmol/L), KN92 (Calbiochem, 500-nmol/L), or GTTI 298 trifluorooacetate salt hydrate (Sigma, 1-µmol/L) were added to cultured cardiomyocytes along with isoproterenol (1-µmol/L) and compared to the appropriate control and
isoproterenol-only groups. In all experiments studying effects of blockers on isoproterenol action, cells from the same isolates were exposed in parallel to isoproterenol as an internal control.

**Electrophysiology**

Cell culture: After 30 hours of exposure to interventions in culture, cardiomyocytes were washed with Tyrode solution. Isoproterenol-treated cells were washed with 1-μmol/L propranolol (Sigma) to block any potential acute effects of residual isoproterenol bound to the membrane. All experiments were performed at 36±1°C. The whole-cell patch clamp technique was used to record currents in voltage-clamp mode. Borosilicate glass electrodes were filled with pipette solution containing (mmol/L): GTP 0.1, K-aspartate 110, MgCl₂ 1, Mg₂-ATP 5, HEPES 10, Na₂-phosphocreatine 5 and EGTA 10; pH adjusted to 7.2 with KOH, and attached to a patch-clamp amplifier (Axopatch 200A). Electrodes had tip-resistance of 2-4 MΩ when filled. To record Iₖₛ, coverslips with cultured cardiomyocytes were placed in the bath and superfused with Tyrode solution containing 1-mmol/L Ca²⁺, CdCl₂ (200-μmol/L, to inhibit L-type Ca²⁺-current) and dofetilide (1-μmol/L, to inhibit Iₖᵢᵣ).

Freshly isolated cells after *in-vivo* treatment: To record Iₖᵢᵣ, ventricular cardiomyocytes were perfused with Tyrode solution containing 1-mmol/L Ca²⁺, CdCl₂ (200-μmol/L, to inhibit L-type Ca²⁺-current) and dofetilide (1-μmol/L, to inhibit Iₖᵢᵣ). Iₖᵢᵣ was defined on the basis of current sensitive to Ba²⁺ (1-mmol/L). For Iₖᵢᵣ recording, the extracellular solution was modified to (mmol/L): N-methylglucamine (NMG) 140; MgCl₂ 1; glucose 5; HEPES 5; HEPESE 10 (pH 7.4, HCl). The whole-cell perforated-patch technique was used to record APs in current-clamp mode. Junction potentials averaged 10.5 mV and were corrected for APs only. The pipette solution for action potential (AP) recordings was modified to contain low EGTA (0.005 mmol/L). The extracellular solution for Ca²⁺-current (Iₖᵢᵣ) measurement contained (mmol/L): tetraethylammonium-chloride 136, CsCl 5.4, MgCl₂ 1, CaCl₂ 2, NaH₂PO₄ 0.33, dextrose 10, and HEPES 5 (pH 7.4, CsOH). Niflumic acid (50 μmol/L) and 4-aminopyridine (2-mmol/L) were added to inhibit Ca²⁺-dependent Cl⁻-current and transient-outward K⁺-current respectively. The pipette solution contained (mmol/L) CsCl 120, tetaethylammonium-chloride 20, MgCl₂ 1, EGTA 10, MgATP 5, HEPESE 10, and Li-GTP 0.1 (pH 7.4, CsOH). Electrodes had tip-resistance of 2-4 MΩ when filled.

Cell capacitance was 104±4 pF for CTL and 106±4 pF for isoproterenol-treated cells in culture. Cell capacitance was 199±11 pF for CTL and 252±16 pF for *in-vivo* isoproterenol-treated animals (P<0.05). Cell capacitance was 146±9 pF for CTL and 180±11 pF for *in-vivo* sp-8p-CPT treated animals (P<0.05).

**Protein-extraction and Immunoblots**

*Protein extraction*: Membrane protein fractions were isolated with extraction buffer containing: 25-mmol/L Tris-HCl (pH 7.34), 5-mmol/L EGTA, 5-mmol/L EDTA, 150-mmol/L NaCl, 0.2 mmol/L Na₃VO₄, 0.1 AEBSF, 20 mmol/L glycerol-2-phosphate, 10-μg/mL aprotinin, 10-μg/mL leupetenin, 1-μmol/L micr oxytcin, 1-μg/mL pepstatin (pH 7.4); followed by homogenization. After centrifugation at 3000 rpm and 4°C for 10 minutes, the supernatant containing the cell membranes was centrifuged at 48,000 rpm for one hour. Membrane pellets were re-suspended in extraction buffer supplemented with 1% Triton X-100 and stored at -20°C.

*Western blots*: Protein concentration was determined with the Bradford method. Membrane protein (10-20 μg) was denatured and fractionated on 8% SDS-polyacrylamide gels, then proteins were transferred electrophoretically to Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore) in 25-mmol/L Tris-base, 192-mmol/L glycine and 20%-ethanol at 0.3 A for 1 hour. Membranes were blocked in phosphate buffered saline (PBS, mmol/L: 137 NaCl, 10 phosphate, 2.7 KCl, pH 7.4, NaOH) with 5% non-fat dry milk for 1 hour and incubated with primary antibodies (rabbit anti-KCNE1 1:1000, as kindly provided by Dr Jacques Barhanin) overnight at 4°C. After washing and re-blocking, membranes were incubated with donkey anti-rabbit (1:10,000, Jackson Immunolabs) secondary antibodies. Antibody was detected with Western-Lightning Chemiluminescence Reagent Plus
(Perkin-Elmer Life Sciences). Later, the same membranes were also probed with anti-GAPDH at room temperature for 1 hour in order to control for equal protein loading. Secondary antibody was horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000, Jackson Immunolabs).

Confocal Microscopy
After 30 hours of exposure to interventions or control vehicle in culture, cardiomyocytes were washed with PBS and then fixed with 2% formaldehyde (20 min, Sigma). Cells were blocked and permeabilized with 2% normal donkey serum (NDS, Jackson), and 0.2% TritonX-100 (Sigma) for 1 hour. Cells were then incubated overnight at 4°C with primary antibodies for KCNE1 (1:200 rabbit polyclonal), NFATc3 (1:200, mouse monoclonal, Santa Cruz) and NFATc4 (1:200, rabbit polyclonal, Santa Cruz) in PBS containing 1% NDS, 1% BSA, 0.05% Triton. This was followed by 3 washes and incubation with secondary antibody (donkey-anti-mouse Alexa-555 and donkey-anti-rabbit Alexa-488, Jackson) at room temperature for 1 hour. Additionally, cells were incubated with ToPro3 (1-µmol/L, Invitrogen) for 30 minutes at room temperature. Confocal microscopy was performed with the Olympus Fluoview FV1000 system. Control experiments with secondary antibodies revealed very low-level or absent background staining. Signals were analyzed with Fluoview Olympus software. Nuclear and cytosolic densities of NFATc3 and NFATc4 staining were determined as the sum of the intensities of pixels within nuclear or cytosolic regions normalized to the corresponding nuclear or cytosolic areas. Phalloidin was used as a cardiomyocyte marker.

RNA Isolation/Real-time PCR
Guinea pig ventricular cardiomyocytes were collected after 30-hr culture in intervention- or CTL-medium, or after isolation following isoproterenol or sp-8pCPT treatment. Total RNA was extracted with Nucleospin RNA II (Macherey- Nagel) kit. Cells were homogenized in TRIzol Reagent (Invitrogen), and mixed with equal amount of 70% ethanol. The lysate was loaded on to a NucleoSpin RNA II column and centrifuged for 30 s at 11,000 g to ensure RNA-binding. MDB (Membrane Desalting Buffer) was added to the columns to prepare the membrane for subsequent DNase reaction. To eliminate genomic DNA, RNA was treated with a DNase reaction mixture (room temperature, 30 min). The silica membrane was washed with Buffer RA2 (wash buffer) and RA3 (wash buffer concentrate) and then dried. RNA was eluted with 40 µL of RNase-free water (HyClone®, Thermo) and centrifuged at 11,000 g for 1 min. RNA was quantified spectrophotometrically at 260-nm and sample integrity was confirmed by agarose gel electrophoresis. One µg of each RNA sample was reverse transcribed with the High Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR was performed with SYBR Green (Power Syber Green master mix, Applied Biosystems). Primer sequences are shown in Supplemental Table 1. Each cDNA sample was run in duplicate on the Stratagene Mx3000P qPCR platform. Primer specificity was verified with dissociation curve analyses and gel electrophoresis of the PCR products. Gene expression levels were normalized to the geometric average of multiple reference genes (18-S rRNA, GAPDH, β-actin and HPRT) according to Vandesompele et al.1

Construction of Epac-1 Knock-down Adenoviral Vectors
To attenuate Epac1 expression in guinea pig cardiomyocytes, an E1-E3-deleted adenoviral vector, over-expressing a microRNA-embedded shRNA (shRNAmir) sequence targeted to Epac1 mRNA (Ensembl Gene ID: ENSCPOG00000006002) was developed. First, we created an adenoviral shuttle plasmid that carries a CMV promoter-driven GFP expression cassette and the microRNA-context sequence in the 3′ untranslated region of GFP with unique restriction sites for cloning of shRNAmir (Supplemental Figure IA). The turbo GFP cDNA was PCR-amplified from pGIPZ (Open Biosystems) with the following primaries: 5′ GGTAGTCGACCACCAGACTCTAGAGGAT (sense) and 5′ TGGCGGCGGCGCGCTACTTGACATTAT (antisense). The PCR product was cloned in pAdTrack-CMV (a gift of Bert Vogelstein, Addgene plasmid #16405) at SalI – NotI sites, generating the AdS-GFP plasmid. Two Xbal fragments of AdS-GFP between positions 1612 and 3298 were deleted using the
dam-, dcm- E. coli strain ER2925 (New England Biolabs), resulting in AdS-GFP-ΔXbaI. Finally, the microRNA-context sequence was PCR amplified from pGIPZ with 5’ TAGCGGCCCGCTTTGTAATGAGGCTTCAG sense and 5’ TGCAAGCTTCGATTAGTCTTC CAATTGAA antisense primers and the PCR product was cloned in AdS-GFP-ΔXbaI between NotI and HindIII sites, resulting in the AdS-empty plasmid. The Epac1-targeted shRNAmir sequence was cloned in AdS-empty (Supplemental Figure IB) following previously published protocols. Briefly, the shRNAmir sequence was designed with the web-based ‘shRNA retriever’ tool available on the homepage of Ravi Sachidanandam’s laboratory (http://katahdin.cshl.org/, Cold Spring Harbor Laboratory, NY, USA). The 97-bp long synthetic oligonucleotides (Epac1: 5’ TGCTGTGACTAGTAGTGAAGCGAACAGAG ATCCTCTAGTAGAAGCCACAGATGTAGTCAGTAGGAATGTCTCTGTCTGCGCTACT GCCTCGGA, scrambled: 5’ TGCTGTGACAGTGACAGTGACAGCGGAACGTAAGGAAAGCGGTGATCTGTAAGCCACAGATGTAGTCAGTAGACTTCTGCCTACTGCCTCGGA, with the 22-bp mature siRNA sequences italicized) were PCR amplified with 5’ CAGAAGGCTCGAGAAGGTTAACTGCTGTGACTAGTGAGCG sense and 5’ CTAAAGTAGCCTCCCTAACTGCCTGAGGACATGTCTCTGTCCTGACTGCTACTGCCTCGGA antisense primers and the PCR products cloned in AdS-empty at XhoI and EcoRI sites. Recombinant adenoviral genomes and initial virus cultures were generated by employing the Adeasy system (Johns Hopkins University, http://www.coloncancer.org/adeasy.htm), according to previously published protocols. Recombinant adenoviruses were amplified in Hek293 cells (ATCC) and were purified with the Adenovirus Standard Purification ViraKit™ (Virapur LLC). Functional titers of the final virus preparations were determined by infecting Hek293T/17 cells with limiting dilutions of the virus. Guinea pig ventricular cardiomyocytes were transduced with the Epac1-KD and scrambled virus and kept in culture for 72 hr with the appropriate controls. The infection efficiency of the virus was close to 100% of viable cardiomyocytes (Supplemental Figure IC).

In vivo chronic β-adrenergic stimulation model
Male Hartley guinea pigs weighing 300-350 g were acclimated to the new environment for a period of one week. Guinea pigs received daily I.P. injections of isoproterenol (Sigma, dissolved in 0.9% saline-solution) for the treatment group and vehicle (0.9% saline-solution) for the control group. Isoproterenol was injected at an initial dose of 50 μg·kg⁻¹·day⁻¹ and after the first week the dose was increased by 100 μg·kg⁻¹·day⁻¹ every week over 13 weeks, to a final dose of 1.4 mg·kg⁻¹·day⁻¹ for the last week. On the last day guinea pigs were anaesthetized with isoflurane (2-3%) and subjected to echocardiography.

In vivo chronic Epac-treatment
Guinea pigs weighing 250-300 g were anaesthetized with isoflurane and osmotic minipumps (model 2006, Alzet, Cupertino, CA) were implanted subcutaneously in the neck. The minipumps were filled with Sp-8-pCPT (Axxora, non-hydrolyzable form of 8-pCPT, dissolved in sterile water), and used to provide constant delivery at 16 μg/day over 6 weeks. Guinea pigs implanted with vehicle-filled minipumps were used as parallel controls.

Echocardiography
Transthoracic echocardiographic studies were performed at baseline and 13 weeks after injection, with animals being sedated with isoflurane, using a phased-array probe 10S (4.5-11.5 Megahertz) in a Vivid 7 dimension system (GE Healthcare Ultrasound, Horten, Norway).

M-mode echocardiograms were used to measure left ventricular (LV) dimensions at both end cardiac diastole (LVDd) and systole (LVDs), LV fractional shortening (FS) was calculated as (LVDd − LVDs) / LVDd X 100%, and LV ejection fraction (EF) was obtained by the formula packed in Vivid 7 dimension system suggested by American Society of Echocardiography. The thickness of LV anterior wall and that of LV posterior wall were also measured in LV M-mode spectrum, LV mass was calculated using formula suggested by Reffelmann et al for small animals.4 LV mass / Body weight (BW), LVDd / BW, and LV mass / LVDd ratio were calculated to evaluate LV structural remodeling.
Pulsed wave Doppler was used to study transmitral flow (TMF), left lower and upper pulmonary venous flow (PVF), and trans aortic flow (TAF). Peak velocity in early filling E wave, time interval from mitral valve (MV) closing to opening (MVco) were measured in TMF. Velocity of systolic flow (S) and that of diastolic flow (D) were measured, and S/D ratio was calculated in PVF. LV ejection time (ET) was measured in TAF. LV global myocardial performance index (MPI) was calculated as (MVco-LVET)/LVET X 100%.

Mitral annulus movement was recorded by tissue Doppler imaging. Lateral and septal mitral annulus moving velocities in systole (S lateral, S septal), early and atrial diastole (e’, a’) were measured. And time intervals from ending of a’ to beginning of e’(b), and from beginning to ending of S (a) were measured. LV regional MPI was calculated by (b-a) / a X 100%, and E/e’ was calculated for both lateral wall and septum.

**Cardiomyocyte T-tubule network analysis**

Freshly isolated ventricular cardiomyocytes were plated on laminin-coated Petri dishes. Cell membranes were stained with 2-μmol/L di-4-ANEPPS in Kraft-Bruhe (KB) solution. Samples were excited with an argon (488nm) laser and fluorescence collected at 515-nm emission wavelength with an LSM 710 confocal microscope. Z-series were acquired every 300-nm from top to bottom of each cardiomyocyte. Fluorescent latex beads (170-nm) were used to determine the point spread function (PSF) of the imaging system. Acquired Z-series were further deconvolved with Huygens Professional 4.4.0 software using maximum likelihood estimation with a Richardson-Lucy algorithm. The 20 most central Z-slices, corresponding to a 6-μm thickness, were used to build maximum intensity projections with MIP rendering. The extent of the T-tubule network was determined using the Image Pro Plus 6.0 software (Media Cybernetics). Briefly, the sum of the pixel intensity associated with the total membrane network was first quantified (a). In a second step, the peripheral membrane region was excluded, considering exclusively the inner membrane network (i.e., T-tubules), and the corresponding sum of the pixel intensity quantified as (b). The extent of T-tubule network within cardiomyocytes (y) was expressed as a percentage of the total membrane network and determined as follows: y = (b x 100)/a.

**Incorporation of [3H]-leucine**

To examine the effect of Isoproterenol on protein synthesis, the incorporation of radioactive-labeled [3H]-leucine was quantified in cardiomyocytes. Cultured guinea pig ventricular cardiomyocytes were stabilized in culture and treated with vehicle (CTL) or isoproterenol (1-μmol/L) in the presence of [3H]-leucine (1-μCi/mL) for 30 h. The cells were washed with PBS and then treated with 10%-trichloroacetic acid at 4°C for 30 min to precipitate protein content. The precipitates were then dissolved in NaOH (0.25 N). Aliquots were counted by liquid scintillation counting.

**Data Analysis**

Clampfit 9.2 (Axon) and GraphPad Prism 5.01 were used for data analysis. Group comparisons were performed with unpaired Student t-tests (for single comparisons between 2 groups) or one-way ANOVA with Bonferroni-corrected t-tests (for multiple-group comparisons). Patch-clamp data were analyzed with two-way ANOVA and Bonferroni-corrected t-tests. A two-tailed P<0.05 indicated statistical significance. Data are expressed as mean±SEM.
References


Supplemental Figures

**Supplemental Figure I.**  
**A:** Schematic illustrating the adenoviral viral construct designed to knock down Epac1 expression in primary culture. CMV=cytomegalovirus promoter GFP=green fluorescent protein  
**B:** Complementary siRNA sequence that was used to knock down Epac1 and corresponding guinea pig Epac1 mRNA sequence.  
**C:** Transmission and corresponding fluorescent mode images of adenovirus-infected ventricular cardiomyocytes.
Supplemental Figure II. A: Mean±SEM $I_{\text{Ks}}$ density-voltage relations at baseline and after 30-hr culture in vehicle-control medium (CTL). B: Representative immunobLOTS and mean±SEM expression-levels for KCNE1 protein following normalization to GAPDH band intensities on the same lanes (n=3). C: Representative immunobLOTS and mean±SEM expression levels for KCNQ1 protein normalized to GAPDH (n=3). Samples were obtained from membrane protein extracts. (N=number of independent experiments).
Supplemental Figure III. A, B: Cell size (cell area, µm²) and cell capacitance (pF) for vehicle (CTL) and isoproterenol-treated cells. C: [³H]-leucine incorporation in cells incubated with vehicle (CTL) or isoproterenol for 30 hours. D: Di-4-ANEPPS T-tubule staining in freshly isolated cardiomyocytes and 30-hour cultured cardiomyocytes in CTL and isoproterenol-containing conditions. E: The extent of T-tubule network within cardiomyocytes analyzed as percentage of the total membrane network (**P<0.01, ***P<0.001, N=number of cells) F: Di-4-ANEPPS T-tubule staining in freshly isolated cardiomyocytes from CTL and isoproterenol treated animals. G: The extent of T-tubule network within cardiomyocytes analyzed as percentage of the total membrane network (N= number of cells).
Supplemental Figure IV. A-D. Effects of in-vitro isoproterenol treatment (for 30 hours) on I_{Ks} properties: A: I_{KSTEP} normalized to maximum values in each cell. B: I_{KTAIL} normalized to maximum value in each cell. Curves are fits to experimental data by Boltzmann function. *P<0.05, **P<0.01, ***P<0.001 vs CTL at the same test potential (TP). (N=number of cells) C: Time constant (τ) of activation as determined with biexponential fits. Isoproterenol significantly accelerated the slow phase time constant. D: Results at +60 mV. E-H. Effects of in-vivo isoproterenol treatment (progressively-increasing doses over 13 weeks) on I_{Ks} properties: E: I_{KSTEP} normalized to maximum values in each cell. F: I_{KTAIL} normalized to maximum value in each cell. Curves are fits to experimental data by Boltzmann function. G: Time constant (τ) of activation as determined with biexponential fits. Isoproterenol significantly accelerated the slow phase time constant. H: Results at +60 mV, *P<0.05, **P<0.01, ***P<0.001 (N=number of cells).
Supplemental Figure V. A: $I_{Ks}$ recordings for control cells and cells acutely perfused with 1-$\mu$mol/L isoproterenol, alone or along with PKI. B: Mean±SEM $I_{Ks}$ density-voltage relations for corresponding conditions. *$P<0.05$, ***$P<0.001$, versus control (CTL).
**Supplemental Figure VI.** *A-C.* *In-vitro* isoproterenol (30-hour exposure in culture) effect on Epac1 and 2 expression. *A, B:* Mean±SEM normalized results for Epac1 (N=11) and Epac2 (N=10) mRNA expression in vehicle (CTL) and isoproterenol treated cells. *C:* Mean±SEM, Epac1/Epac2 ratio in CTL and isoproterenol treated cells. *D-F.* *In-vivo* isoproterenol effect (progressively-increasing doses over 13 weeks) on Epac1 and Epac2 expression. *D, E:* Mean±SEM normalized results for Epac1 and Epac2 mRNA expression in CTL (N=5) and isoproterenol (N=7) treated animals. *F:* Mean±SEM, Epac1/Epac2 ratio in CTL and isoproterenol treated cells.
Supplemental Figure VII. Mean±SEM normalized results for Epac1 and Epac2 expression in the presence of GFP-carrying adenovirus in a bicistronic vector with a scrambled construct (Scr+Iso), GFP-carrying adenovirus in a bicistronic vector with an Epac-knockdown probe (KD+Iso), and Iso-alone (Iso). Cells were exposed to isoproterenol for 30 hours at the end of a 72-hour period virus-incubation period. ***P<0.001. C, D: Mean±SEM normalized results for KCNE1 and KCNQ1 mRNA expression in the presence of Scr+ isoproterenol, KD + isoproterenol and Iso-alone. *P<0.05
Supplemental Figure VIII. A, B: Mean±SEM normalized results for Epac1 and Epac2 mRNA expression in scrambled construct (Scr) and Scr+isoproterenol (Iso) treated cells. Cells were exposed to isoproterenol or vehicle for 30 hours at the end of a 72-hour period virus-incubation period. *$P<0.05$. 
Supplemental Figure IX. Effects of Epac-knockdown on isoproterenol-induced NFAT-localization changes. Immunolocalization of NFATc3 and NFATc4 viral gene-transfer cells in isoproterenol-cultured cardiomyocytes for scrambled (Scr+Iso) or Epac1 knockdown (KD+Iso) virus. Top: Representative images. Bottom: Mean±SEM ratios of nuclear/cytosolic NFATc3 (**P<0.01) and NFATc4 (*P<0.05) fluorescence-intensity ratios. N=number of cells. Because of the limited number of cells in each heart, the fact that cells were divided for different conditions, and the substantial death-rate of cells in prolonged culture with viral infection, a very limited number of healthy surviving cells was available after immunostaining for each condition. We therefore analyzed only 1 cell per experiment, and the Ns shown are the number of experiments. Cells were exposed to isoproterenol for 30 hours at the end of a 72-hour period virus-incubation period.
Supplemental Figure X. Effects of in-vivo isoproterenol administration (progressively increasing doses over 13 weeks) on cellular electrophysiological properties. A: Representative $I_{CaL}$ recordings from CTL and isoproterenol treated animals. B: Mean±SEM $I_{CaL}$ density *$P<0.05$, **$P<0.01$, ***$P<0.001$ (n=number of cells) C: Representative $I_{K1}$ recordings from CTL and isoproterenol treated animals D: Mean±SEM $I_{K1}$ density-voltage relations, recorded as Ba$^{2+}$-sensitive current, with ramp protocol in insert. Please note different scales for CTL versus Iso recordings in C. *$P<0.05$, **$P<0.01$, ***$P<0.001$ (n=number of cells).
**Supplemental Figure XI.** Effects of in-vivo sp-8-pCPT administration on cellular electrophysiological properties. Guinea pig were treated with 16 μg/day sp-8-pCPT or vehicle for 6 weeks, after which cardiomyocytes were isolated and subjected to patch-clamp study. 

A: cell capacitance in CTL and sp-8-pCPT condition (pF) (*P<0.05, n=number of cells). 

B: HW/BW ratio in CTL and sp-8-pCPT treated animals (n=number of animals). 

C: Representative I_{CaL} recordings from CTL and sp-8-pCPT treated animals. 

D: Means±SEM I_{CaL} density, *P<0.05. 

E: Representative I_{K1} recordings from CTL and sp-8-pCPT treated animals, recorded as Ba^{2+}-sensitive current, with ramp protocol in insert. 

F: Means±SEM I_{K1} density-voltage relations *P<0.05, ***P<0.001 (n=number of cells).
Predicted NFAT binding sites within the 5k KCNE1 guinea pig promoter

-474 GCTCAACTAT TCTCAAGGAAA GAGACATTTAT GGTAGCTACT GTTATATCG
  NFAT binding site
-454 ATCAGGGTAT AAAACACTGT GGAAGCAAGGA AGACGTCCTG AGGACAGGAG
  NFAT binding site
-429 TCGAATCTAG TATTTTTGAT TTTCACTTTAA GTGAGAAACT CTCTCCCTAAA
  NFAT binding site
-429 ATGTCTCTGTT TGCCGGAGTG GGGACAAGAG GTGAACAAGGA GAATCTCTCAA
-349 CTGGAAGGCT CTGCTGGCCCT CAGTTGGCCT ATTTCTCTGAT AAGAGGTAAA
  NFAT binding site
-349 TAGCATATGT GTCCAGAAATT TGGACAGAGG GTGTAGAGAAAG ATGAAAGAAC
-339 AACAGGAATGT GCTTCTCGAA AGAAACTGAC TCATCGGATT CTGGACCTAA
-334 TAGTAGGATCAT TACGCCCAAAAT TTAGGCTCTA CATAATGGTT TTTACCTCTAG
-329 TGCTGGTTCC TAAAACCAAT AATGCCCAAAT GATTTCAAGA CTGACATCTTT
  NFAT binding site
-309 GGGCGTGGACCT CACCCCTCGG GCCCGGTCCT CCGAAGATAT CCCCCCTCCG
-304 GAGGGAAACTGT GGGCTCAACA TCAAAAGGCC TTGCCAGGGC AGTAAACACCA
  NFAT binding site
-299 TAAGGAAAGG CAAAAGTTCG TGAGCTGCTG AAGACGCTGTT TTTGGGAGGC
  NFAT binding site
-299 TTCCCCCTGGCT TTATCACTGTG TGATAGGTGTA GCAAACCTTAA GCAGACACTA
-349 AGACATCTGTA GAGCCAGTGT CACCCACGTTT CCACGAGGAG ACAGGCGACT TAAACCATCT
-299 ACAACCTCCTT ATCTCTGTGCT TAATAATTTCT TAACTAGGTG GAAAACACAA
  NFAT binding site
-249 TAAAGTCATC CTGGAACCAC AGAATGTGCG CCTGCTATGT CCGTCTGACC
-199 TGGCAAGATCT GTGATTTAGCT CATTTCTCCA AAAAGAAAT GACGAGAGG
-149 AGAGGAATCTA TTCTGCTGCA TCTGGCTCCA TTGCCCTCTT CTTGCTTGCT
-99 GTCCGTGTGT GTCAGGTGTGT GTCAGGTGTGT GTCAGGTGTGT
  TSS (+1) r+
-49 ATTTTAGCCCAA TCTGTGACTTT CTGATTTTCG AGCTGCACTTT TCTTTTCTCCG
+2 AGCTTGCTTGGGACGCTTG CTTGCCACCG TGAAGCTCTG CAGGCGCAGCA
+52 TGAGCTGTATGAA TAAATTTGAC TGTGGGAGTCC CCTTCTCGAC CAGGGGTGG
  Translation Start Site
+102 CAGGGGAGAG AGCTTAACAA

Supplemental Figure XII. Putative NFAT binding sites for KCNE1 promoter in guinea pig. 5000 bp-length sequence upstream of the transcription start site (translation start site is in yellow highlight) of guinea pig KCNE1 promoter. Gene analyzed by MatInspector (Genomatix, www.genomatix.de).
Supplemental Figure XIII. Left: $I_{Ks}$-recordings (step to +50 mV) and Right: Mean±SEM, $I_{Ks}$ density-voltage relations in cultured cells for: A, B: CTL cardiomyocytes versus isoproterenol (1-µmol/L, 30-hr) treated cardiomyocytes versus GGTTI+isoproterenol treated cells. Rap1 inhibition prevented isoproterenol-induced decrease in $I_{Ks}$ density. C, D: CTL cardiomyocytes versus isoproterenol-only versus U73122+isoproterenol exposed cardiomyocytes. PLC inhibition did not prevent isoproterenol-induced decrease in $I_{Ks}$ density. E, F: CTL cardiomyocytes versus isoproterenol treated cells, isoproterenol + KN93-treated cells and isoproterenol + KN92-treated treated cells. All exposures were for 30 hours during culture. CaMKII inhibition prevented isoproterenol-mediated reduction in $I_{Ks}$ density. *P<0.05, **P<0.01, ***P<0.001, versus control at same voltage. (n= number of cells).
Supplemental Figure XIV: 

A: I_{Ks}-recordings (step to +50 mV) and B: Mean±SEM, I_{Ks} density-voltage relations in cultured cells treated with Rap1 inhibitor (GGTI) or CaMKII inhibitor (KN93) or vehicle, in the absence of adrenergic stimulation.
## Supplemental Tables

### Supplemental Table I.

<table>
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<th>Gene name:</th>
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<th>Gene Bank no/ID</th>
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<tr>
<td><strong>RAPGEF3</strong> (Epac1)</td>
<td>For 5' GATGTGGAAGCGAAGACCAT</td>
<td>ENSCPOG 00000006002</td>
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<td></td>
<td>Rev 5' AGGGTGTAACGCAGCAAGAT</td>
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<tr>
<td>RAPGEF4 (Epac2)</td>
<td>For 5' GAGCTGGTGAGACTGGATGCT</td>
<td>ENSCPOG 00000010605</td>
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<td></td>
<td>Rev 5' TGGTTGAGGACACCGTCCTCT</td>
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<tr>
<td><strong>KCNE1</strong></td>
<td>For 5' TCGCAGCGACCGTT</td>
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<td></td>
<td>Rev 5' TCAATGACGCACACGATCTG</td>
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<td><strong>KCNQ1</strong></td>
<td>For 5' TCAGGCCGCATGACTTTT</td>
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<td></td>
<td>Rev 5' GATTCGCACCATGAGTTGA</td>
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<tr>
<td><strong>HPRT1</strong></td>
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<td>ENSCPOG0000002512</td>
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<td>Rev 5' CCTCCCATCCTCTGATCACAT</td>
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<td><strong>β-actin</strong></td>
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<td>Rev 5' AGGGTGTAACGCAGCAAGAT</td>
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<td><strong>GAPDH</strong></td>
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<td></td>
<td>Rev 5' TCTGGGTGGCAGTGATG</td>
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<tr>
<td><strong>18S</strong></td>
<td>For 5' ACGGCTACCACATCAAGGAGA</td>
<td>NT_176398.1</td>
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<td>Rev 5' CCAATATTACAGGGCCTCGAA</td>
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Supplemental Table II. General and Echocardiographic Indices of Left Ventricular Structural Remodeling

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<th></th>
<th>Control (n=5)</th>
<th>Iso (n=9)</th>
<th>( P ) value (Iso vs control)</th>
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<tr>
<td><strong>LVDd (mm)</strong></td>
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<tr>
<td>Baseline</td>
<td>7.56±0.75</td>
<td>7.75±0.40</td>
<td>0.539</td>
</tr>
<tr>
<td>End</td>
<td>10.54±0.51</td>
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<tr>
<td>% change</td>
<td>40.6±16.1</td>
<td>45.1±8.6</td>
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<tr>
<td><strong>LVDd/BW Ratio (mm/kg)</strong></td>
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<tr>
<td>Baseline</td>
<td>23.9±2.1</td>
<td>23.7±2.2</td>
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<td>End</td>
<td>10.7±1.0</td>
<td>12.8±1.7</td>
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<td>% change</td>
<td>-55.0±3.6</td>
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<td><strong>LV mass (g)</strong></td>
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<td>Baseline</td>
<td>0.84±0.09</td>
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<tr>
<td>End</td>
<td>1.92±0.30</td>
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<tr>
<td>% change</td>
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<td><strong>LV mass/BW Ratio (g/kg)</strong></td>
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<td>2.65±0.22</td>
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<td>% change</td>
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<td>18.0±16.8</td>
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<td><strong>LV mass/LVDd Ratio (g/mm)</strong></td>
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<tr>
<td>Baseline</td>
<td>0.11±0.00</td>
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<td>End</td>
<td>0.18±0.02</td>
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<tr>
<td>% change</td>
<td>63.7±17.8</td>
<td>119.1±30.4</td>
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LVDd: left ventricular dimension at end cardiac diastole; BW: body weight.
### Supplemental Table III. Echocardiographic Indices of Left Ventricular Systolic Function

<table>
<thead>
<tr>
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<tr>
<td><strong>FS (%)</strong></td>
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<tr>
<td>Baseline</td>
<td>42.7±6.9</td>
<td>39.9±3.7</td>
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<tr>
<td>End</td>
<td>48.8±6.4</td>
<td>35.3±4.1</td>
<td>0.002</td>
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<tr>
<td>% change</td>
<td>11.0±30.4</td>
<td>-11.1±12.9</td>
<td>0.077</td>
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<td><strong>EF (%)</strong></td>
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<tr>
<td>Baseline</td>
<td>78.4±7.0</td>
<td>75.8±4.1</td>
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<td>End</td>
<td>81.0±6.3</td>
<td>69.2±5.5</td>
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<td>% change</td>
<td>4.4±16.2</td>
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<tr>
<td><strong>S\textsubscript{Septal} (cm/s)</strong></td>
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<tr>
<td>Baseline</td>
<td>3.34±1.18</td>
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<td>End</td>
<td>4.46±1.04</td>
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<td>% change</td>
<td>38.9±24.6</td>
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FS: left ventricular (LV) fractional shortening; EF: LV ejection fraction; S\textsubscript{Septal}: LV basal septal systolic contractility.
## Supplemental Table IV. Echocardiographic Indices of Left Ventricular Diastolic Function

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<td><strong>Lateral E/e’ Ratio</strong></td>
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<td>End</td>
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<tr>
<td><strong>Septal E/e’ Ratio</strong></td>
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<td>Baseline</td>
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<td>11.3±2.7</td>
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<td>% change</td>
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<td>Baseline</td>
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<td>% change</td>
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<tr>
<td>% change</td>
<td>1.3±25.3</td>
<td>-28.8±26.2</td>
<td>0.066</td>
</tr>
</tbody>
</table>

Lateral E/e’: transmitral flow E wave velocity/lateral mitral annulus moving velocity; Septal E/e’: transmitral flow E wave velocity/septal mitral annulus moving velocity; S/D: systolic wave velocity / diastolic wave velocity ratio; PVF: pulmonary venous flow.
Supplemental Table V. Echocardiographic Indices of Left Ventricular Myocardial Performance

<table>
<thead>
<tr>
<th></th>
<th>Control (n=5)</th>
<th>Iso (n=9)</th>
<th>P value (Iso vs Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Global MPI (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>42.7±7.3</td>
<td>39.4±6.9</td>
<td>0.409</td>
</tr>
<tr>
<td>End</td>
<td>40.8±8.2</td>
<td>50.0±11.6</td>
<td>0.146</td>
</tr>
<tr>
<td>% change</td>
<td>-1.6±28.3</td>
<td>30.5±39.8</td>
<td>0.139</td>
</tr>
<tr>
<td><strong>Lateral MPI (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>46.6±7.8</td>
<td>40.5±5.8</td>
<td>0.117</td>
</tr>
<tr>
<td>End</td>
<td>45.9±12.1</td>
<td>55.7±6.5</td>
<td>0.067</td>
</tr>
<tr>
<td>% change</td>
<td>-2.4±13.1</td>
<td>40.1±25.6</td>
<td>0.004</td>
</tr>
<tr>
<td><strong>Septal MPI (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>41.2±4.6</td>
<td>37.5±7.5</td>
<td>0.343</td>
</tr>
<tr>
<td>End</td>
<td>39.4±9.2</td>
<td>56.7±8.8</td>
<td>0.004</td>
</tr>
<tr>
<td>% change</td>
<td>-4.0±19.2</td>
<td>55.4±32.0</td>
<td>0.002</td>
</tr>
</tbody>
</table>

MPI: myocardial performance index.