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

Mona Aflaki, Xiao-Yan Qi, Ling Xiao, Balazs Ordog, Artavazd Tadevosyan, Xiaobin Luo, Ange Maguy, Yanfen Shi, Jean-Claude Tardif, Stanley Nattel

Rationale: **β-Adrenoceptor 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 (I_{Ks}).**

Objective: **To assess the molecular mechanisms of I_{Ks} 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 I_{Ks} 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-adrenoceptor antagonist 1-[2-((3-Carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol dihydrochloride (CGP-20712A) prevented isoproterenol-induced I_{Ks} downregulation, whereas the β_2-antagonist ICI-118551 had no effect. The selective Epac activator 8-pCPT-2′-O-Me-cAMP decreased I_{Ks} density to an extent similar to isoproterenol exposure, and adenoviral-mediated knockdown of Epac1 prevented isoproterenol-induced I_{Ks}/KCNE1 downregulation. In contrast, protein kinase A inhibition with a cell-permeable highly selective peptide blocker did not affect I_{Ks} 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 I_{Ks} reduction by isoproterenol and INCA6 suppressed isoproterenol-induced KCNE1 downregulation, consistent with signal-transduction via the Ca^{2+}/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 I_{Ks} density and KCNE1 mRNA and protein expression while inducing cardiac dysfunction and action potential prolongation. Selective in vivo activation of Epac via sp-8-pCPT-2′-O-Me-cAMP infusion decreased I_{Ks} density and KCNE1 mRNA/protein expression.**

Conclusions: **Prolonged β_1-adrenoceptor stimulation suppresses I_{Ks} by downregulating KCNE1 mRNA and protein via Epac-mediated Ca^{2+}/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.[1–3] Plasma norepinephrine concentration elevation predicts outcomes in CHF,[4] and β-blockers reduce CHF mortality.[5] Reduced slow delayed-rectifier potassium K^+-current (I_{Ks}) is a particularly common and important finding in CHF-related remodeling.[3,6] Reduced I_{Ks} 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-

Original received November 5, 2013; revision received February 6, 2014; accepted February 7, 2014. In January 2014, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 14.35 days.

From the Department of Medicine, Research Center, Montreal Heart Institute, Université de Montréal, Montreal, Quebec, Canada (M.A., X.-Y.Q., L.X., B.O., A.T., X.L., A.M., Y.S., J.-C.T., S.N.); and Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec, Canada (M.A., S.N.).

The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.113.302982/-/DC1.

Correspondence to Stanley Nattel, MD, Department of Pharmacology, McGill University, 5000 Belanger St E, Montreal, Quebec H1T 1C8, Canada. E-mail stanley.nattel@icm-mhi.org

Nonstandard Abbreviations and Acronyms

8-pCPT	8-pCPT-2′-O-Me-cAMP
AP	action potential
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetate
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase type II
CGP-20712A	1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol dihydrochloride
CHF	congestive heart failure
Epac	exchange protein directly activated by cAMP
Forskolin	7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one
GGTI 298	N-[4-[2(R)-amino-3-mercaptopropyl]amino-2-(1-naphthalenyl)benzoyl]-L-leucine methyl ester trifluoroacetate salt
ICI-118551	(±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride
I_{CaL}	L-type calcium current
I_{K1}	inward rectifier current
I_{Ks}	slow delayed-rectifier potassium current
KCNE1	potassium voltage-gated channel subfamily E member 1
KCNQ1	potassium voltage-gated channel, KQT-like subfamily, member 1
KN93	N-[2-[N-(4-Chlorocinnamyl)-N-methylaminomethyl]phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulfonamide phosphate salt
NFAT	nuclear factor of activated T cells
PKA	protein kinase A
PKI	protein kinase A inhibitor peptide
Rap1	Ras-related protein 1

sium voltage-gated channel, KQT-like subfamily, member 1 (KCNQ1) 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 areflexic coma followed by 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 Kraftbrühe 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)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol dihydrochloride (CGP-20712A); (±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride (ICI-118551); 8-Br-cAMP; 7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one (forskolin); 8-pCPT-2′-O-Me-cAMP (8-pCPT); inhibitor of nuclear factor of activated T cell (NFAT)-calcineurin association-6 (NFAT6); cyclosporine; 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetate acetoxymethyl 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 ($[^3H]$-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±11pF 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 Immunochemistry

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. Immunochemistry 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 changes[14] 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 I_{Ks}

We first established the stability of the guinea pig cell culture system in vitro (Online Figure II). I_{Ks} 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 IIIB), and increased leucine incorporation (Online Figure IIIC). T-tubule density decreased in culture, with significantly greater decreases in isoproterenol-treated cells versus parallel controls (Online Figure IIID and IIIE), potentially accounting for unchanged capacitance in isoproterenol-treated cells, despite increased cell size.

I_{Ks} 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

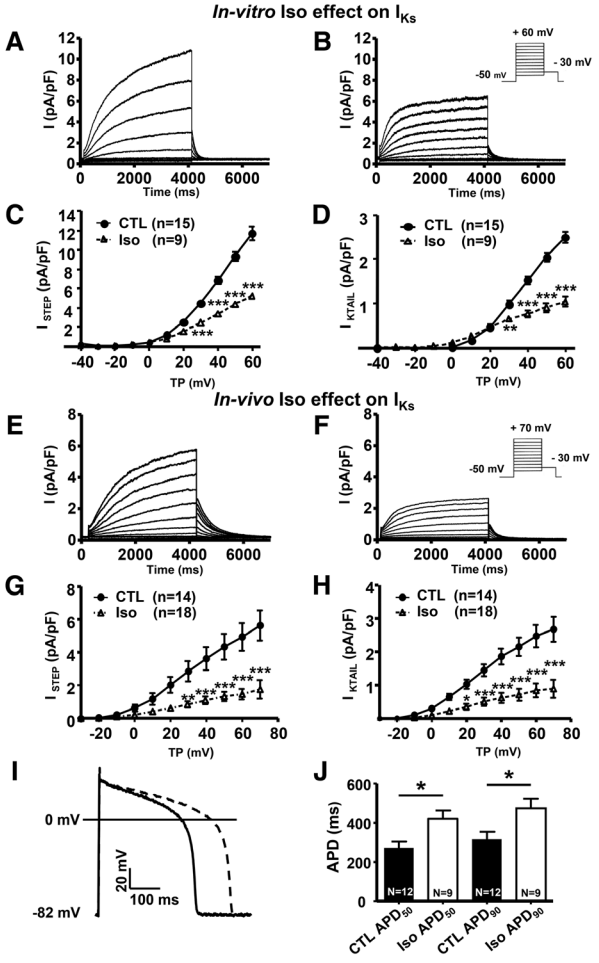

Figure 1. Effects of isoproterenol (Iso) on slow delayed-rectifier potassium current (I_{Ks}) and action potentials (APs). **A** to **D**, In vitro effects. **A** and **B**, Original recordings in cells incubated with control and isoproterenol containing medium, respectively. Voltage protocol (4-s depolarizing pulses at 0.1 Hz, followed by 3 seconds at –30 mV to observe tail currents). **C**, I_{Kstep} density–voltage relations for cells cultured in the presence of Iso or vehicle (control [CTL]). **D**, Tail current (I_{KTAIL}) density–voltage relations. **E** to **H**, Effects of chronic in vivo isoproterenol administration on I_{Ks}. **E** and **F**, Original recordings in cells incubated with control and isoproterenol containing medium, respectively. **G**, I_{Kstep} density–voltage relations in freshly isolated ventricular cardiomyocytes from isoproterenol-treated and CTL animals. **H**, Tail current (I_{KTAIL}) density–voltage relations. **I**, Representative AP recordings (1 Hz) from guinea pigs treated with isoproterenol and CTL animals. **J**, AP duration (APD) at 50% (APD50) and 90% (APD90) repolarization. ***P*<0.01, ****P*<0.001 vs CTL at same test potential (TP). Group data are mean±SEM. n indicates number of cells.

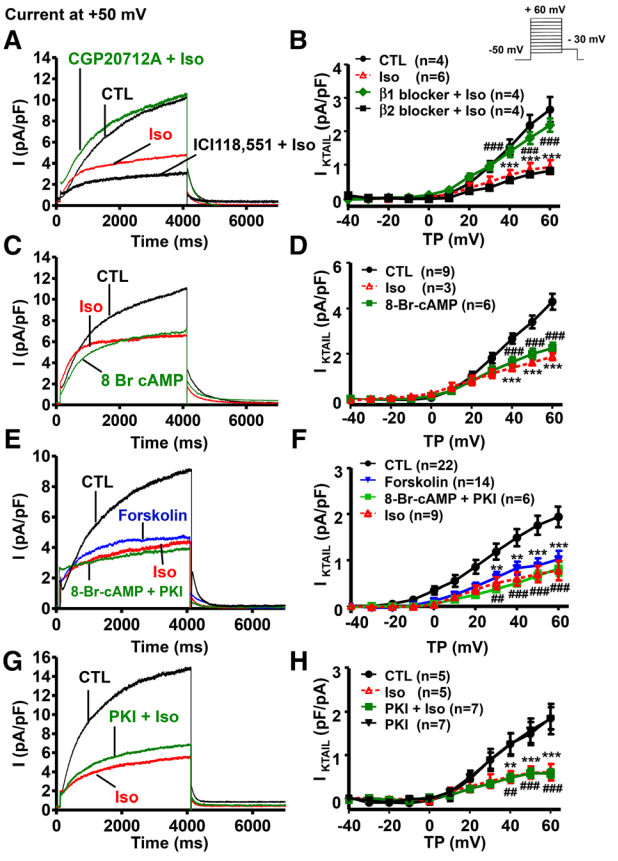

Figure 2. Relationship between isoproterenol (Iso) effects and β-receptor subtype, cAMP and protein kinase A inhibitor peptide (PKI). Left, Slow delayed-rectifier potassium current (I_{Ks}) recordings (+50 mV) and **right**, mean±SEM data; under vehicle-culture (control [CTL]) and (**A** and **B**) Iso, 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-18551]) 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-trihydroxylabd-14-en-11-one (forskolin), 8-Br-cAMP+PKI (****P*<0.001, forskolin vs CTL; ###*P*<0.001, 8-Br-cAMP+PKI vs CTL). **G** and **H**, PKI (****P*<0.001, Iso vs CTL; ##*P*<0.01, ###*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_{Ks} activation by reducing the slow-phase time constant (Online Figure IVC and IVD).

Involvement of β_1-Adrenoceptors and cAMP Signaling

Cells incubated with isoproterenol and highly selective β1 (CGP-20712A) or β2 (ICI-118551) antagonists were compared with parallel control and isoproterenol-alone groups. Figure 2A shows representative I_{Ks} recordings. Corresponding current density/voltage relationships (Figure 2B) indicate that concomitant treatment with the β_1-blocker CGP-20712A abolished the isoproterenol effect. The β_2-blocker ICI-118551 failed to alter isoproterenol action, confirming that the isoproterenol effect is mediated through β_1-adrenergic receptors.

After β_1-adrenoceptor activation, the trimeric G-protein complex releases $G_{\alpha s}$, which activates adenylyl cyclase, increasing intracellular cAMP levels.[15] Sustained exposure to the cell-permeable cAMP agonist 8-bromo-cAMP reduced I_{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_{Ks} enhancement caused by β-adrenergic stimulation is mediated by PKA activation/phosphorylation of KCNQ1 on Ser-27.[16] To evaluate the role of PKA in I_{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 μmol/L). PKI did not suppress isoproterenol-induced I_{Ks} downregulation (Figure 2G and 2H). In contrast, PKI blunted I_{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_{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-μmol/L 8-pCPT-2′-O-Me-cAMP (8-pCPT), a highly selective Epac activator.[17] Sustained Epac activation with 8-pCPT reduced I_{Ks} densities to values comparable with those in a

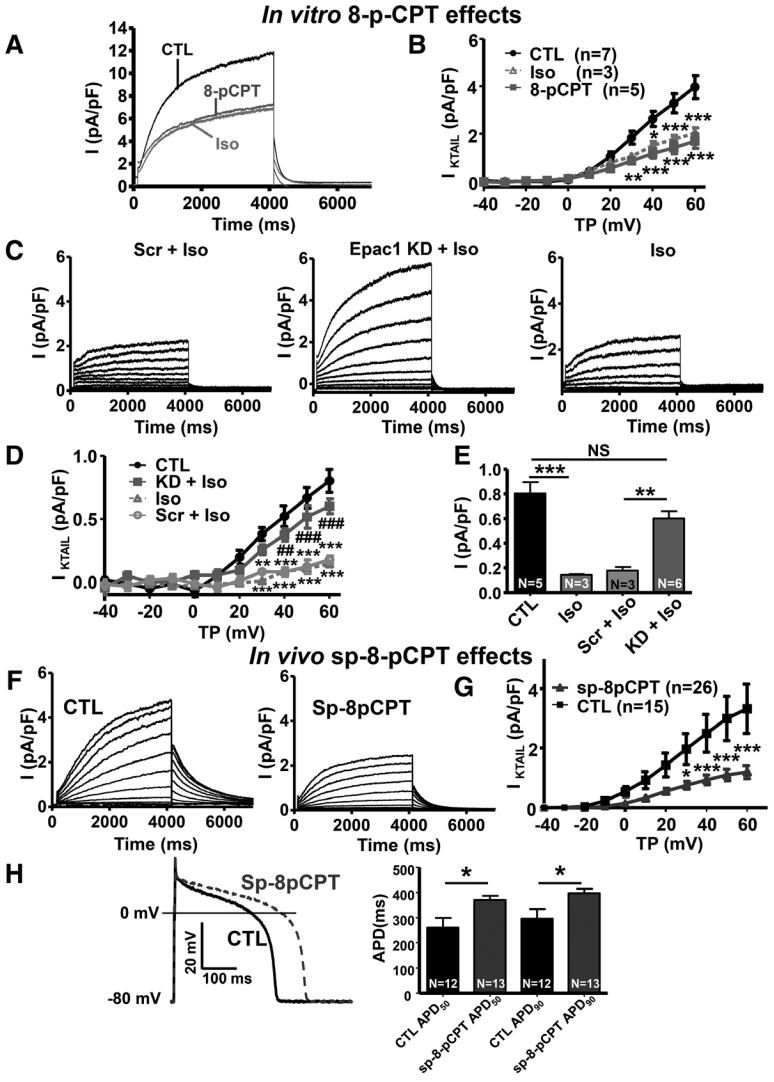

Figure 3. Effects of isoproterenol (Iso), exchange protein directly activated by cAMP (Epac) activation and Epac knockdown. A to **E**, In vitro studies. **A**, Slow delayed-rectifier potassium current (I_{Ks}) recordings at +50 mV under vehicle-control (CTL), Iso, and 8-pCPT-2′-O-Me-cAMP. **B**, Mean±SEM I_{Ks} density–voltage relations. **C**, I_{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**, Mean±SEM I_{Ks} density–voltage relations $^{**}P<0.01$, $^{***}P<0.001$ vs CTL; ##$P<0.01$, ###$P<0.001$ vs Iso-alone. **E**, Mean±SEM I_{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_{Ks} recordings from animals treated with sp-8-pCPT and vehicle (CTL). **G**, Mean±SEM tail current (I_{KTAIL}) 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 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 VIIB). The scrambled-virus did not alter Epac expression in the presence of isoproterenol (Online Figure VIIA), and isoproterenol significantly increased Epac1 expression in the presence of scrambled-virus versus scrambled-virus incubation alone (Online Figure VIIIA). 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.[21] 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 translocation as an event 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 IIIG). 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 VID and VIE), but the increase in Epac2 was larger than in Epac1, decreasing the Epac1/Epac2 expression ratio (Online Figure VIF). In vivo isoproterenol also remodeled other ionic currents, reducing L-type calcium current (I_{CaL}) density by ≈45% and inward rectifier current (I_{K1}) by ≈47% (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).

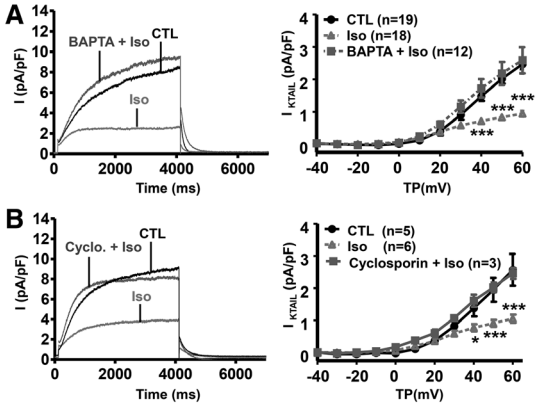

Figure 4. Effects of intracellular Ca^{2+} buffering and calcineurin inhibition. A, **Left**, Slow delayed-rectifier K^+ current (I_{Ks}) recordings (at +50 mV) after culture in vehicle-control (CTL), isoproterenol (Iso) or 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetate acetoxymethyl ester (BAPTA)-AM plus Iso (BAPTA-AM+Iso). **Right**, Corresponding mean±SEM I_{Ks} 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 relationship. *$P<0.05$, ***$P<0.001$ vs CTL. n indicates number of cells; and TP, test potential.

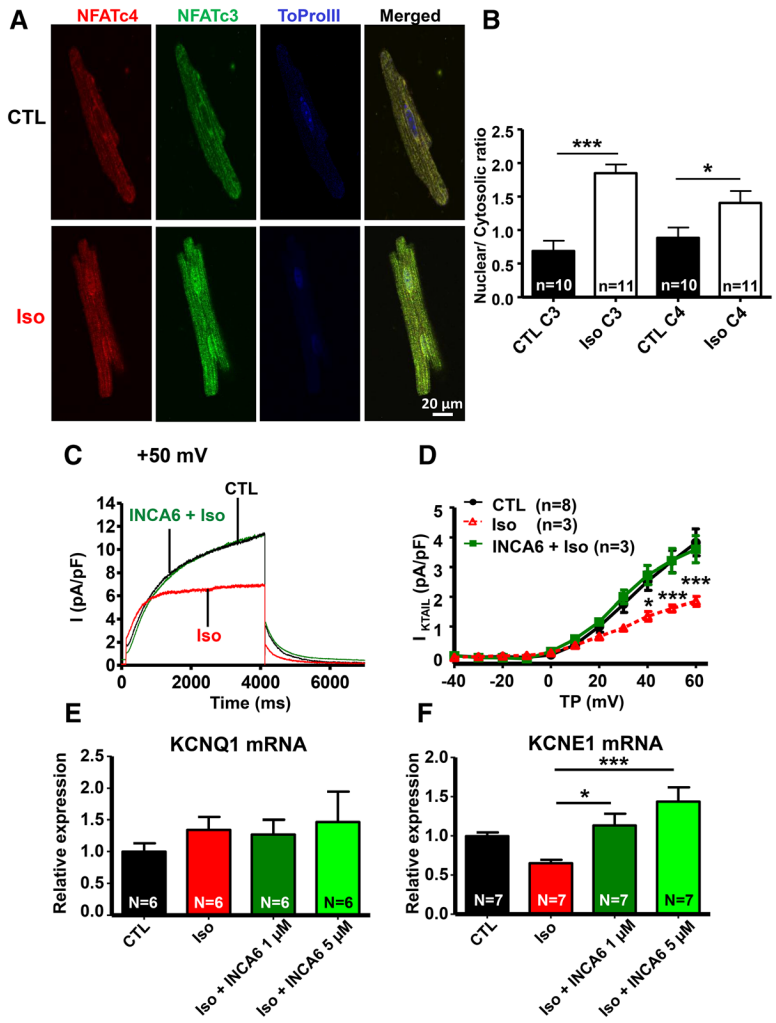

Figure 5. Role of nuclear factor of activated T cells (NFAT) in isoproterenol effects.
A, Immunolocalization of NFATc3 and NFATc4 in cardiomyocytes cultured in vehicle-control (CTL) or isoproterenol-containing (Iso) medium. ToProIII was used to label nuclei. **B**, Mean±SEM nuclear/cytosolic NFATc3 and NFATc4 fluorescence-intensity ratios. *P<0.05, ***P<0.001 vs CTL. **C**, I_{Ks} recordings (at +50 mV) from cells cultured under CTL, Iso, and Iso+INCA6 (1 µmol/L) conditions. Voltage protocol as in Figure 1. **D**, Mean±SEM I_{Ks} density–voltage relations in CTL, Iso, and inhibitor of NFAT-calcineurin association-6 (INCA6; 1 µmol/L)+Iso. *P<0.05, ***P<0.001 vs CTL. **E** and **F**, Mean±SEM potassium voltage-gated channel, KQT-like subfamily, member 1 (KCNQ1) and potassium voltage-gated channel subfamily E member 1 (KCNE1) mRNA expression in cells cultured with control-vehicle, isoproterenol-alone, and isoproterenol in the presence of INCA6 (1 or 5 µmol/L). *P<0.05, ***P<0.001 for comparison shown (for **E** and **F**, number (n) of independent quantitative polymerase chain reaction analyses, each with RNA from cultured cells from 2 hearts). N indicates number of cells; and TP, test potential.

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 ≈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 ≈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 β_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 XIIIE and XIIIF), 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 β-adrenergic stimulation decreases I_{Ks} density both in vitro and in vivo while

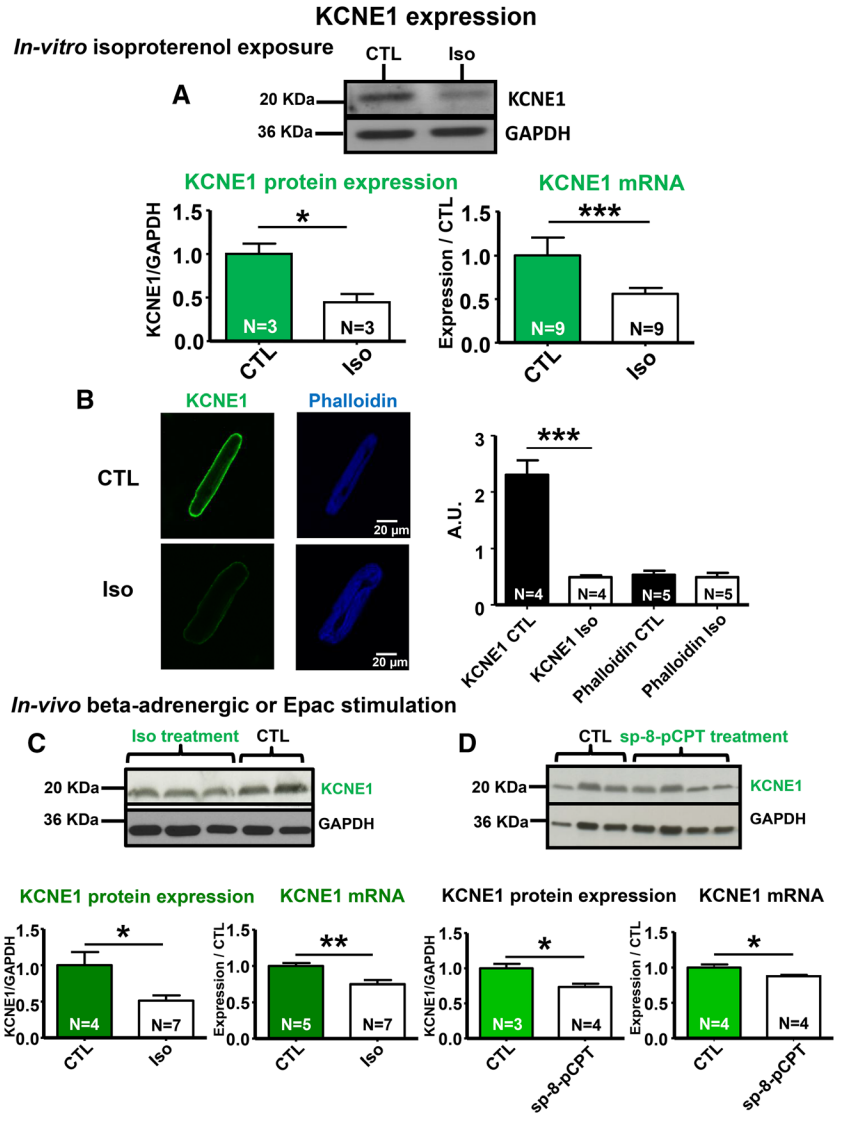

Figure 6. Potassium voltage-gated channel subfamily E member 1 (KCNE1) expression changes. A and **B**, In vitro studies. **A**, **Top**, Crude membrane protein extracts and RNA extracts were obtained from cells cultured in control-vehicle (CTL) and isoproterenol-medium (Iso). KCNE1 bands were seen on immunoblot at the expected molecular mass of ≈20 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**, Mean±SEM protein expression levels (**P*<0.05); **right**, mRNA expression. **D**, **Top**, Immunoblots for membrane KCNE1 protein for animals treated with sp-8-pCPT or vehicle. **Bottom**, **Left**, Mean±SEM protein expression levels; **right**, mRNA expression (**P*<0.05, ****P*<0.001). n indicates numbers of independent experiments, each from 1 heart.

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_{Ks} downregulation occurs in patients with terminal CHF[1] and in ventricular and atrial cells from different animal models.[3,24–30] Animal models of hypertrophy also show reduced I_{Ks}.[31] Atrioventricular block–induced remodeling also decreases I_{Ks} in ventricular cardiomyocytes.[32,33] Less is known about the signal-transduction mechanisms that lead to I_{Ks} downregulation and the underlying changes in I_{Ks} 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[30] 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.[35]

Epac Signaling in Cardiac Remodeling

cAMP, the universal second messenger that is produced via adenylyl cyclase after β-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 β_1-adrenergic receptors activates Epac1, which decreases I_{Ks} 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.[36] Myocardial Epac1 expression increases in rats with pressure-overload induced by aortic constriction and in rat

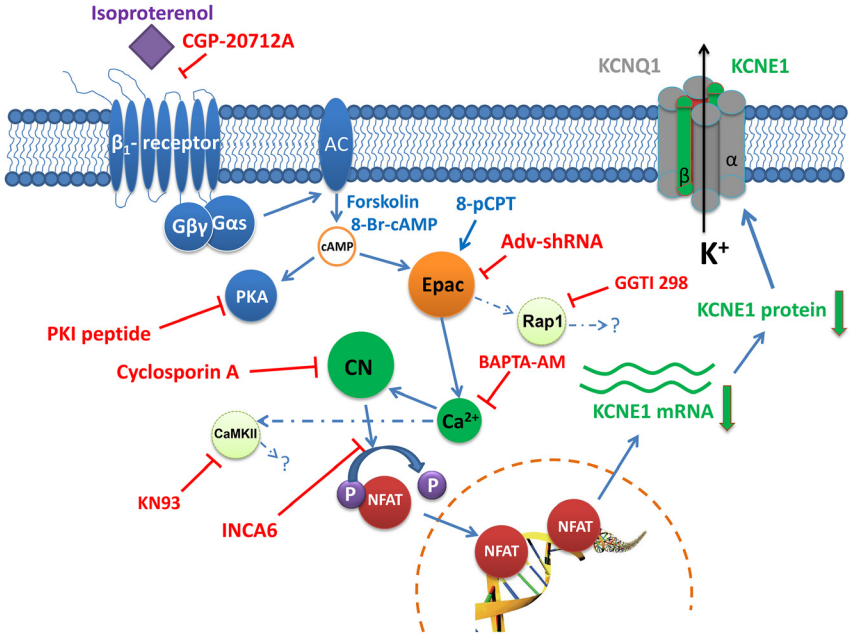

Figure 7. Schematic representation of the mechanisms involved in slow delayed-rectifier K⁺ current (I_{Ks}) downregulation by sustained β-adrenergic stimulation. Blockers (red) and activators (blue) were used to probe specific components of the pathway. 8-pCPT indicates 8-pCPT-2′-O-Me-cAMP; AC, adenylyl 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-carbamoyl-4-hydroxy)phenoxy)ethylamino]-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; and Rap1, Ras-related protein 1.

ventricular cardiomyocytes treated with isoproterenol.[10] Epac1 and Epac2 are also upregulated in the hearts of mice subjected to chronic-isoproterenol infusion.[37] Epac1 expression is increased ≈2-fold in ventricular cardiomyocytes from patients with CHF, with no change in Epac2 expression.[10] 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.[20] Little is known about the role of Epac in cardiac electrophysiology. Epac activation inhibits ATP-sensitive K⁺-channels in pancreatic β-cells[38] and Epac1 coimmunoprecipitates with SUR1, a subunit of the K_{ATP}-channel. Exposure of rat chromaffin cells to 8-pCPT increases T-type Ca^{2+}-current and $Ca_v3.1$-subunit expression.[39] Acute perfusion of rat and mouse cardiomyocytes with 8-pCPT does not affect L-type Ca^{2+} current,[23,40] but the Epac activator 8-4-(chlorophenylthio)-2'-*O*-methyladenosine-3',5'-monophosphate (cpTOME) strongly enhances Ca^{2+}-induced Ca^{2+} release in mouse cardiomyocytes.[22] Acute Epac activation failed to induce any changes in AP duration in 2 studies[40,41]; however, a more recent investigation showed AP duration increases in rats after acute 8-CPT-acetoxymethyl-ester perfusion.[42]

Epac activation increases Ca^{2+} sparks via CaMKII phosphorylation of ryanodine receptors in rat cardiomyocytes.[40] A recent elegant study showed that in vivo infusion of an Epac activator to rats elicits a PKA-independent positive inotropic response, increases cardiomyocyte Ca^{2+} transients, enhances sarcoplasmic reticulum Ca^{2+} stores and Ca^{2+} transients, and promotes Ca^{2+}-dependent arrhythmic activity.[43] Inhibition of calcineurin or CaMKII prevented Epac-induced Ca^{2+} responses.

The present study is the first to implicate Epac in I_{Ks} remodeling. The Epac dependence of adrenergically induced I_{Ks} 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 I_{Ks} and KCNE1 expression when Epac was knocked down. Signaling was Ca^{2+} 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 I_{Ks} and KCNE1 downregulation, and the suppression of isoproterenol-induced I_{Ks} 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,[10] and Epac activation is known to significantly increase NFAT nuclear translocation,[44] which is important for the induction of cardiac hypertrophy.

Relationship to Other Signaling Systems in K⁺ Channel Remodeling

Rossow et al[45] have shown spatial heterogeneity of NFATc3-dependent I_{to} downregulation, causing a loss of the normal transmural gradient in mouse ventricular cardiomyocytes after chronic in vivo isoproterenol infusion. β-Adrenergic stimulation increased intracellular Ca^{2+}, calcineurin, and NFAT activity, which reduced Kv4.2 expression and I_{to} density.[45] The upstream pathway was not identified. In mice with myocardial infarction, downregulation of I_{to} and $I_{Kslow1,2}$ was prevented by calcineurin inhibition or NFATc3 knockout.[46] Although calcineurin/NFAT signaling suppresses I_{to} transcription in most studies,[47,48] I_{to} upregulation occurs in neonatal rat cardiomyocytes.[49] Cav1.2 is downregulated via the same pathway in canine cardiomyocytes.[50] NFAT is an important downstream mediator of responses to changes in intracellular Ca^{2+}[51]; 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 I_{Ks} regulation. Previous studies have identified a role for CaMKII in I_{to} downregulation in tachycardia remodeling of canine ventricular cardiomyocytes.[48] Interestingly, in that work, like the present study, the primary signaling system involved was the Ca^{2+}/calcineurin/NFAT system, but intact CaMKII function was necessary for K⁺ current downregulation. CaMKII activation also suppresses

delayed-rectifier K^+ currents in neurons.[52] Transgenic CaMKIIδc overexpression reduces Kir2.1 expression and I_{K1} in mice.[53] CaMKII-expression is increased in calcineurin-transgenic mice; CaMKII-inhibitory drugs improve left ventricular function and prevent arrhythmias.[54] Less is known about the role of Rap1 in cardiac electrophysiology. Rap1, along with phospholipase C, participates in Ca^{2+}-induced Ca^{2+}-release after β-adrenergic stimulation and Epac activation.[22] It is possible that CaMKII and Rap1 contribute to Ca^{2+} liberation, which we found was essential for I_{Ks} downregulation. Prior studies have demonstrated a role for Rap1 and CaMKII in Epac-induced increases of murine Ca^{2+}-induced Ca^{2+} release, although intact phospholipase C was also needed.[22] 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_{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^+ channel expression. Our findings may be relevant to the prevention of malignant arrhythmias in a variety of contexts. Sympatho-adrenergic activation is an important contributor to arrhythmic risk in patients with CHF,[4] as well as in animal models.[55] 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.[56] 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_{Ks} through chronically elevated background adrenergic tone. The Epac system was described relatively recently,[18] and our knowledge about its role in cardiac pathophysiology is rather limited.[57] 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_{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_{Ks} channels, slowing activation and enhancing current density.[58] 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^+ 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_{Ks} density and associated subunits in culture. In addition, the density of I_{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_{Ks} properties among species.[59,60] 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,[61] 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_{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_{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^+ current (60% decrease in I_{Ks}) more than inward Ca^{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_{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.[62] We based the Epac regimen on prior studies in rats, which used continuous infusion for 4 weeks,[63] 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,[10,17,20,22,36,38,40–44] but sp-8-pCPT products can have effects on other signaling systems.[64] We confirmed the role of Epac signaling in vitro with adenoviral-mediated knockdown; however, we were unable to apply gene knockdown in vivo; this limitation should be considered in interpreting our results.

Acknowledgments

We thank Nathalie L'Heureux, Chantal St. Cyr, Marc-Andre Meus, and Louis-Robert Villeneuve for technical help and France Thériault for secretarial help.

Sources of Funding

This work was supported by Canadian Institutes of Health Research (MOP68929), Quebec Heart and Stroke Foundation and Fondation Leducq (ENAFRA award, 07CVD03).

Disclosures

None.

References

1. Beuckelmann DJ, Näbauer M, Erdmann E. Alterations of K+ currents in isolated human ventricular myocytes from patients with terminal heart failure. *Circ Res*. 1993;73:379–385.
2. Kääb S, Nuss HB, Chiamvimonvat N, O'Rourke B, Pak PH, Kass DA, Marban E, Tomaselli GF. Ionic mechanism of action potential prolongation in ventricular myocytes from dogs with pacing-induced heart failure. *Circ Res*. 1996;78:262–273.
3. Nattel S, Maguy A, Le Bouter S, Yeh YH. Arrhythmogenic ion-channel remodeling in the heart: heart failure, myocardial infarction, and atrial fibrillation. *Physiol Rev*. 2007;87:425–456.
4. Cohn JN, Levine TB, Olivari MT, Garberg V, Lura D, Francis GS, Simon AB, Rector T. Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure. *N Engl J Med*. 1984;311:819–823.
5. Shibata MC, Flather MD, Wang D. Systematic review of the impact of beta blockers on mortality and hospital admissions in heart failure. *Eur J Heart Fail*. 2001;3:351–357.
6. Akar FG, Wu RC, Juang GJ, Tian Y, Burysek M, Disilvestre D, Xiong W, Armoundas AA, Tomaselli GF. Molecular mechanisms underlying K+ current downregulation in canine tachycardia-induced heart failure. *Am J Physiol Heart Circ Physiol*. 2005;288:H2887–H2896.
7. Splawski I, Shen J, Timothy KW, Lehmann MH, Priori S, Robinson JL, Moss AJ, Schwartz PJ, Towbin JA, Vincent GM, Keating MT. Spectrum of mutations in long-QT syndrome genes. KVLQT1, HERG, SCN5A, KCNE1, and KCNE2. *Circulation*. 2000;102:1178–1185.
8. Zhang LM, Wang Z, Nattel S. Effects of sustained beta-adrenergic stimulation on ionic currents of cultured adult guinea pig cardiomyocytes. *Am J Physiol Heart Circ Physiol*. 2002;282:H880–H889.
9. Bos JL. Epac proteins: multi-purpose cAMP targets. *Trends Biochem Sci*. 2006;31:680–686.
10. Métrich M, Lucas A, Gastineau M, Samuel JL, Heymes C, Morel E, Lezoualc'h F. Epac mediates beta-adrenergic receptor-induced cardiomyocyte hypertrophy. *Circ Res*. 2008;102:959–965.
11. Paddison PJ, Cleary M, Silva JM, Chang K, Sheth N, Sachidanandam R, Hannon GJ. Cloning of short hairpin RNAs for gene knockdown in mammalian cells. *Nat Methods.* 2004;1:163–167.
12. Luo J, Deng ZL, Luo X, Tang N, Song WX, Chen J, Sharff KA, Luu HH, Haydon RC, Kinzler KW, Vogelstein B, He TC. A protocol for rapid generation of recombinant adenoviruses using the AdEasy system. *Nat Protoc.* 2007;2:1236–1247.
13. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. 2002;3:RESEARCH0034.
14. Reffelmann T, Kloner RA. Transthoracic echocardiography in rats. Evalution of commonly used indices of left ventricular dimensions, contractile performance, and hypertrophy in a genetic model of hypertrophic heart failure (SHHF-Mcc-facp-Rats) in comparison with Wistar rats during aging. *Basic Res Cardiol*. 2003;98:275–284.
15. Rockman HA, Koch WJ, Lefkowitz RJ. Seven-transmembrane-spanning receptors and heart function. *Nature*. 2002;415:206–212.
16. Marx SO, Kurokawa J, Reiken S, Motoike H, D'Armiento J, Marks AR, Kass RS. Requirement of a macromolecular signaling complex for beta adrenergic receptor modulation of the KCNQ1-KCNE1 potassium channel. *Science*. 2002;295:496–499.
17. Christensen AE, Selheim F, de Rooij J, Dremier S, Schwede F, Dao KK, Martinez A, Maenhaut C, Bos JL, Genieser HG, Døskeland SO. cAMP analog mapping of Epac1 and cAMP kinase. Discriminating analogs demonstrate that Epac and cAMP kinase act synergistically to promote PC-12 cell neurite extension. *J Biol Chem*. 2003;278:35394–35402.
18. de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature*. 1998;396:474–477.
19. Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, Housman DE, Graybiel AM. A family of cAMP-binding proteins that directly activate Rap1. *Science*. 1998;282:2275–2279.
20. Métrich M, Berthouze M, Morel E, Crozatier B, Gomez AM, Lezoualc'h F. Role of the cAMP-binding protein Epac in cardiovascular physiology and pathophysiology. *Pflugers Arch*. 2010;459:535–546.
21. Hogan PG, Chen L, Nardone J, Rao A. Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev*. 2003;17:2205–2232.
22. Oestreich EA, Malik S, Goonasekera SA, Blaxall BC, Kelley GG, Dirksen RT, Smrcka AV. Epac and phospholipase Cepsilon regulate Ca2+ release in the heart by activation of protein kinase Cepsilon and calcium-calmodulin kinase II. *J Biol Chem*. 2009;284:1514–1522.
23. Yoo B, Lemaire A, Mangmool S, Wolf MJ, Curcio A, Mao L, Rockman HA. Beta1-adrenergic receptors stimulate cardiac contractility and CaMKII activation in vivo and enhance cardiac dysfunction following myocardial infarction. *Am J Physiol Heart Circ Physiol*. 2009;297:H1377–H1386.
24. Tsuji Y, Zicha S, Qi XY, Kodama I, Nattel S. Potassium channel subunit remodeling in rabbits exposed to long-term bradycardia or tachycardia: discrete arrhythmogenic consequences related to differential delayed-rectifier changes. *Circulation*. 2006;113:345–355.
25. Li GR, Lau CP, Ducharme A, Tardif JC, Nattel S. Transmural action potential and ionic current remodeling in ventricles of failing canine hearts. *Am J Physiol Heart Circ Physiol*. 2002;283:H1031–H1041.
26. Tsuji Y, Opthof T, Kamiya K, Yasui K, Liu W, Lu Z, Kodama I. Pacing-induced heart failure causes a reduction of delayed rectifier potassium currents along with decreases in calcium and transient outward currents in rabbit ventricle. *Cardiovasc Res*. 2000;48:300–309.
27. Li D, Melnyk P, Feng J, Wang Z, Petrecca K, Shrier A, Nattel S. Effects of experimental heart failure on atrial cellular and ionic electrophysiology. *Circulation*. 2000;101:2631–2638.
28. Li GR, Lau CP, Leung TK, Nattel S. Ionic current abnormalities associated with prolonged action potentials in cardiomyocytes from diseased human right ventricles. *Heart Rhythm*. 2004;1:460–468.
29. Petkova-Kirova PS, Gursoy E, Mehdi H, McTiernan CF, London B, Salama G. Electrical remodeling of cardiac myocytes from mice with heart failure due to the overexpression of tumor necrosis factor-alpha. *Am J Physiol Heart Circ Physiol*. 2006;290:H2098–H2107.
30. Rose J, Armoundas AA, Tian Y, DiSilvestre D, Burysek M, Halperin V, O'Rourke B, Kass DA, Marbán E, Tomaselli GF. Molecular correlates of altered expression of potassium currents in failing rabbit myocardium. *Am J Physiol Heart Circ Physiol*. 2005;288:H2077–H2087.
31. Furukawa T, Kurokawa J. Potassium channel remodeling in cardiac hypertrophy. *J Mol Cell Cardiol*. 2006;41:753–761.
32. Volders PG, Sipido KR, Vos MA, Kulcsár A, Verduyn SC, Wellens HJ. Cellular basis of biventricular hypertrophy and arrhythmogenesis in dogs with chronic complete atrioventricular block and acquired torsade de pointes. *Circulation*. 1998;98:1136–1147.
33. Ramakers C, Vos MA, Doevendans PA, Schoenmakers M, Wu YS, Scicchitano S, Iodice A, Thomas GP, Antzelevitch C, Dumaine R. Coordinated down-regulation of KCNQ1 and KCNE1 expression contributes to reduction of I(Ks) in canine hypertrophied hearts. *Cardiovasc Res*. 2003;57:486–496.
34. Borlak J, Thum T. Hallmarks of ion channel gene expression in end-stage heart failure. *FASEB J*. 2003;17:1592–1608.
35. Vrtovec B, Delgado R, Zewail A, Thomas CD, Richartz BM, Radovancevic B. Prolonged QTc interval and high B-type natriuretic peptide levels together predict mortality in patients with advanced heart failure. *Circulation*. 2003;107:1764–1769.
36. Métrich M, Morel E, Berthouze M, Pereira L, Charron P, Gomez AM, Lezoualc'h F. Functional characterization of the cAMP-binding proteins Epac in cardiac myocytes. *Pharmacol Rep*. 2009;61:146–153.
37. Ulucan C, Wang X, Baljinnyam E, Bai Y, Okumura S, Sato M, Minamisawa S, Hirotani S, Ishikawa Y. Developmental changes in gene expression of Epac and its upregulation in myocardial hypertrophy. *Am J Physiol Heart Circ Physiol*. 2007;293:H1662–H1672.
38. Kang G, Chepurny OG, Malester B, Rindler MJ, Rehmann H, Bos JL, Schwede F, Coetzee WA, Holz GG. cAMP sensor Epac as a determinant of ATP-sensitive potassium channel activity in human pancreatic beta cells and rat INS-1 cells. *J Physiol.* 2006;573:595–609.
39. Novara M, Baldelli P, Cavallari D, Carabelli V, Giancippoli A, Carbone E. Exposure to cAMP and beta-adrenergic stimulation recruits Ca(V)3 T-type channels in rat chromaffin cells through Epac cAMP-receptor proteins. *J Physiol.* 2004;558:433–449.
40. Pereira L, Métrich M, Fernández-Velasco M, Lucas A, Leroy J, Perrier R, Morel E, Fischmeister R, Richard S, Bénitah JP, Lezoualc'h F, Gómez

AM. The cAMP binding protein Epac modulates Ca2+ sparks by a Ca2+/calmodulin kinase signalling pathway in rat cardiac myocytes. *J Physiol*. 2007;583:685–694.
41. Hothi SS, Gurung IS, Heathcote JC, Zhang Y, Booth SW, Skepper JN, Grace AA, Huang CL. Epac activation, altered calcium homeostasis and ventricular arrhythmogenesis in the murine heart. *Pflugers Arch*. 2008;457:253–270.
42. Brette F, Blandin E, Simard C, Guinamard R, Sallé L. Epac activator critically regulates action potential duration by decreasing potassium current in rat adult ventricle. *J Mol Cell Cardiol*. 2013;57:96–105.
43. Ruiz-Hurtado G, Domínguez-Rodríguez A, Pereira L, Fernández-Velasco M, Cassan C, Lezoualc'h F, Benitah JP, Gómez AM. Sustained Epac activation induces calmodulin dependent positive inotropic effect in adult cardiomyocytes. *J Mol Cell Cardiol*. 2012;53:617–625.
44. Morel E, Marcantoni A, Gastineau M, Birkedal R, Rochais F, Garnier A, Lompré AM, Vandecasteele G, Lezoualc'h F. cAMP-binding protein Epac induces cardiomyocyte hypertrophy. *Circ Res*. 2005;97:1296–1304.
45. Rossow CF, Dilly KW, Yuan C, Nieves-Cintrón M, Cabarrus JL, Santana LF. NFATc3-dependent loss of I(to) gradient across the left ventricular wall during chronic beta adrenergic stimulation. *J Mol Cell Cardiol*. 2009;46:249–256.
46. Rossow CF, Minami E, Chase EG, Murry CE, Santana LF. NFATc3-induced reductions in voltage-gated K+ currents after myocardial infarction. *Circ Res*. 2004;94:1340–1350.
47. Rossow CF, Dilly KW, Santana LF. Differential calcineurin/NFATc3 activity contributes to the Ito transmural gradient in the mouse heart. *Circ Res*. 2006;98:1306–1313.
48. Xiao L, Coutu P, Villeneuve LR, Tadevosyan A, Maguy A, Le Bouter S, Allen BG, Nattel S. Mechanisms underlying rate-dependent remodeling of transient outward potassium current in canine ventricular myocytes. *Circ Res*. 2008;103:733–742.
49. Gong N, Bodi I, Zobel C, Schwartz A, Molkentin JD, Backx PH. Calcineurin increases cardiac transient outward K+ currents via transcriptional up-regulation of Kv4.2 channel subunits. *J Biol Chem*. 2006;281:38498–38506.
50. Qi XY, Yeh YH, Xiao L, Burstein B, Maguy A, Chartier D, Villeneuve LR, Brundel BJ, Dobrev D, Nattel S. Cellular signaling underlying atrial tachycardia remodeling of L-type calcium current. *Circ Res*. 2008;103:845–854.
51. Dolmetsch RE, Lewis RS, Goodnow CC, Healy JI. Differential activation of transcription factors induced by Ca2+ response amplitude and duration. *Nature*. 1997;386:855–858.
52. Sun C, Sumners C, Raizada MK. Chronotropic action of angiotensin II in neurons via protein kinase C and CaMKII. *Hypertension*. 2002;39:562–566.
53. Wagner S, Hacker E, Grandi E, Weber SL, Dybkova N, Sossalla S, Sowa T, Fabritz L, Kirchhof P, Bers DM, Maier LS. Ca/calmodulin kinase II differentially modulates potassium currents. *Circ Arrhythm Electrophysiol*. 2009;2:285–294.
54. Khoo MS, Li J, Singh MV, Yang Y, Kannankeril P, Wu Y, Grueter CE, Guan X, Oddis CV, Zhang R, Mendes L, Ni G, Madu EC, Yang J, Bass M, Gomez RJ, Wadzinski BE, Olson EN, Colbran RJ, Anderson ME. Death, cardiac dysfunction, and arrhythmias are increased by calmodulin kinaseII in calcineurin cardiomyopathy. *Circulation*. 2006;114:1352–1359.
55. Sabbah HN, Goldberg AD, Schoels W, Kono T, Webb C, Brachmann J, Goldstein S. Spontaneous and inducible ventricular arrhythmias in a canine model of chronic heart failure: relation to haemodynamics and sympathoadrenergic activation. *Eur Heart J*. 1992;13:1562–1572.
56. Schwartz PJ. Pharmacological and non-pharmacological management of the congenital long QT syndrome: the rationale. *Pharmacol Ther*. 2011;131:171–177.
57. Laurent AC, Breckler M, Berthouze M, Lezoualc'h F. Role of Epac in brain and heart. *Biochem Soc Trans*. 2012;40:51–57.
58. Romey G, Attali B, Chouabe C, Abitbol I, Guillemare E, Barhanin J, Lazdunski M. Molecular mechanism and functional significance of the MinK control of the KvLQT1 channel activity. *J Biol Chem*. 1997;272:16713–16716.
59. Lu Z, Kamiya K, Opthof T, Yasui K, Kodama I. Density and kinetics of I(Kr) and I(Ks) in guinea pig and rabbit ventricular myocytes explain different efficacy of I(Ks) blockade at high heart rate in guinea pig and rabbit: implications for arrhythmogenesis in humans. *Circulation*. 2001;104:951–956.
60. Zicha S, Moss I, Allen B, Varro A, Papp J, Dumaine R, Antzelevich C, Nattel S. Molecular basis of species-specific expression of repolarizing K+ currents in the heart. *Am J Physiol Heart Circ Physiol*. 2003;285:H1641–H1649.
61. Pavlović D, McLatchie LM, Shattock MJ. The rate of loss of T-tubules in cultured adult ventricular myocytes is species dependent. *Exp Physiol.* 2010;95:518–527.
62. Soltysinska E, Olesen SP, Osadchii OE. Myocardial structural, contractile and electrophysiological changes in the guinea-pig heart failure model induced by chronic sympathetic activation. *Exp Physiol.* 2011;96:647–663.
63. Ruiz-Hurtado G, Domínguez-Rodríguez A, Pereira L, Fernández-Velasco M, Cassan C, Lezoualc'h F, Benitah JP, Gómez AM. Sustained Epac activation induces calmodulin dependent positive inotropic effect in adult cardiomyocytes. *J Mol Cell Cardiol*. 2012;53:617–625.
64. Poppe H, Rybalkin SD, Rehmann H, Hinds TR, Tang XB, Christensen AE, Schwede F, Genieser HG, Bos JL, Doskeland SO, Beavo JA, Butt E. Cyclic nucleotide analogs as probes of signaling pathways. *Nat Methods.* 2008;5:277–278.

Novelty and Significance

What Is Known?

- Chronic adrenergic hyperactivity characterizes a variety of arrhythmic conditions, including congestive heart failure.
- Although the acute arrhythmogenic effects of adrenergic stimulation are well defined, the effects of chronic adrenergic stimulation on the electrophysiological determinants of arrhythmia are less clear.
- Sustained adrenergic activation has been shown to downregulate slow delayed-rectifier K^+-current, I_{Ks}, but the molecular mechanisms are poorly defined.

What New Information Does This Article Contribute?

- Sustained β-adrenergic stimulation reduces I_{Ks} and delays repolarization by causing transcriptional downregulation of the β-subunit potassium voltage-gated channel subfamily E member 1.
- These effects are mediated via the exchange protein activated by cAMP and not by protein kinase A.
- Exchange protein activated by cAMP acts by initiating a Ca^{2+}-calmodulin/calcineurin/nuclear factor of activated T cells signaling pathway.

Cardiac rhythm disturbances (arrhythmias) remain a leading cause of death for many cardiac diseases (like congestive heart failure) that are associated with a sustained increase in sympathetic nervous system adrenergic drive. It is important to know the molecular mechanisms by which arrhythmias occur to devise more effective preventive approaches. The mechanisms by which a transient increase in adrenergic stimulation causes arrhythmias are well known. However, how sustained adrenergic hyperactivity alters cardiac electric function to make the heart vulnerable to dangerous rhythm disturbances is poorly understood. In this study, we asked how sustained β-adrenergic stimulation causes arrhythmia-promoting downregulation of important cardiac potassium channels. With the use of in vitro experiments with guinea pig heart cells and in vivo studies involving long-term administration of β-adrenergic signaling agonists to guinea pigs in vivo, we defined the underlying basis: a specific pathway mediated by the exchange protein activated by cAMP, which increases intracellular calcium activation of calcineurin/nuclear factor of activated T cells signaling to transcriptionally downregulate an accessory subunit of a cardiac potassium channel known to be important in maintaining cardiac electric stability. These findings have the potential to help in devising innovative approaches to preventing sudden cardiac death.