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Phosphoinositide 3-Kinase γ Protects Against Catecholamine-Induced Ventricular Arrhythmia Through Protein Kinase A–Mediated Regulation of Distinct Phosphodiesterases

Alessandra Ghigo, PhD, **Alessia Perino, PhD**, **Hind Mehel, BSc**, **Alexandra Zahradníková Jr, PhD**, **Fulvio Morello, MD, PhD**, **Jérôme Leroy, PhD**, **Viacheslav O. Nikolaev, PhD**, **Federico Damilano, PhD**, **James Cimino, BSc**, **Elisa De Luca, PhD**, **Wito Richter, PhD**, **Ruth Westenbroek, PhD**, **William A. Catterall, PhD**, **Jin Zhang, PhD**, **Chen Yan, PhD**, **Marco Conti, MD**, **Ana Maria Gomez, PhD**, **Grégoire Vandecasteele, PhD**, **Emilio Hirsch, PhD***, and **Rodolphe Fischmeister, PhD***

Department of Genetics, Biology, and Biochemistry, Molecular Biotechnology Center, University of Torino, Torino, Italy (A.G., A.P., F.D., J.C., E.D.L., E.H.); INSERM UMR-S 769, LabEx LERMIT, Châtenay-Malabry, France (H.M., A.Z., J.L., A.M.G., G.V., R.F.); Université Paris-Sud, Faculté de Pharmacie, Châtenay-Malabry, France (H.M., A.Z., J.L., A.M.G., G.V., R.F.); San Giovanni Battista Hospital, Torino, Italy (F.M.); Emmy Noether Group of the DFG, Department of Cardiology and Pneumology, Georg August University Medical Center, Göttingen, Germany (V.O.N.); Department of Obstetrics, Gynecology and Reproductive Sciences, University of California San Francisco, San Francisco, CA (W.R., M.C.); University of Washington School of Medicine, Department of Pharmacology, Seattle, WA (R.W., W.A.C.); Johns Hopkins University School of Medicine, Baltimore, MD (J.Z.); and University of Rochester Medical Center, Rochester, NY (C.Y.). Dr Damilano is currently at the Beth Israel Deaconess Medical Center, Boston, MA. Dr Zahradniková is currently on leave from Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Bratislava, Slovakia

Abstract

Background—Phosphoinositide 3-kinase γ (PI3Kγ) signaling engaged by β-adrenergic receptors is pivotal in the regulation of myocardial contractility and remodeling. However, the role of PI3Kγ in catecholamine-induced arrhythmia is currently unknown.

Methods and Results—Mice lacking PI3Kγ (PI3K$\gamma^{-/-}$) showed runs of premature ventricular contractions on adrenergic stimulation that could be rescued by a selective β_2-adrenergic receptor blocker and developed sustained ventricular tachycardia after transverse aortic constriction. Consistently, fluorescence resonance energy transfer probes revealed abnormal cAMP accumulation after β_2-adrenergic receptor activation in PI3K$\gamma^{-/-}$ cardiomyocytes that depended on the loss of the scaffold but not of the catalytic activity of PI3Kγ. Downstream from β-adrenergic receptors, PI3Kγ was found to participate in multiprotein complexes linking protein kinase A to the activation of phosphodiesterase (PDE) 3A, PDE4A, and PDE4B but not of PDE4D. These PI3Kγ-regulated PDEs lowered cAMP and limited protein kinase A–mediated phosphorylation of

Correspondence to Emilio Hirsch, PhD, Molecular Biotechnology Center, University of Torino, Via Nizza 52, 10126 Torino, Italy (emilio.hirsch@unito.it); or Rodolphe Fischmeister, PhD, INSERM UMRS-769, Université Paris-Sud, Faculté de Pharmacie, 5, Rue J.-B. Clément, F-92296 Châtenay-Malabry Cedex, France (rodolphe.fischmeister@inserm.fr).
*Drs Hirsch and Fischmeister contributed equally to this article.

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L-type calcium channel ($Ca_v1.2$) and phospholamban. In PI3K$\gamma^{-/-}$ cardiomyocytes, $Ca_v1.2$ and phospholamban were hyperphosphorylated, leading to increased Ca^{2+} spark occurrence and amplitude on adrenergic stimulation. Furthermore, PI3K$\gamma^{-/-}$ cardiomyocytes showed spontaneous Ca^{2+} release events and developed arrhythmic calcium transients.

Conclusions—PI3Kγ coordinates the coincident signaling of the major cardiac PDE3 and PDE4 isoforms, thus orchestrating a feedback loop that prevents calcium-dependent ventricular arrhythmia.

Keywords

arrhythmias, cardiac; class II phosphatidylinositol 3-kinases; 3′,5′-cyclic-AMP phosphodiesterases; cyclic AMP-dependent protein kinases; receptors, adrenergic beta-2

Ventricular arrhythmia is a leading cause of death in ischemic heart disease and heart failure and in otherwise healthy individuals.[1] Arrhythmogenesis can be linked to deregulation of the β-adrenergic receptor (β-AR)/cAMP/protein kinase A (PKA) pathway.[2,3] β-ARs are G protein–coupled receptors that primarily trigger $G_{\alpha s}$, which promotes adenylyl cyclase activity and cAMP production.[4] In turn, cAMP-mediated activation of PKA evokes phosphorylation of effectors modulating the cardiac excitation-contraction coupling such as the L-type Ca^{2+} channel (LTCC), the ryanodine receptor (RyR), phospholamban, and troponin I.[4]

Clinical Perspective on p 2083

The spatial and temporal compartmentalization of cAMP ensures that PKA encounters its substrates in the right place and at the right time.[5] On agonist stimulation, cAMP does not increase globally. Rather, cAMP is produced in discrete microdomains, thereby initiating defined sets of PKA-mediated events.[6] For instance, the 2 main cardiac β-AR isoforms, β_1-AR and β_2-AR,[7] signal through the common cAMP/PKA pathway, but β_1-ARs are much more efficient in enhancing cardiac contractility than β_2-ARs.[8] This is due in part to a differential localization of β-AR subtypes, which leads to compartment-restricted cAMP generation.[9,10]

cAMP compartmentalization is also mediated by A-kinase anchoring proteins, which anchor PKA and phosphodiesterases (PDEs) in defined compartments, thus directing localized cAMP destruction.[11,12] Among cardiac cAMP PDEs,[13] PDE3 and PDE4 provide the main route for cAMP degradation and limit cAMP generated by β_2-ARs.[14,15] Disruption of selected and localized subsets of these PDEs has been linked to arrhythmogenesis. In mouse models, defective PDE4D activity in the sarcoplasmic reticulum (SR) RyR complex[2] or defective PDE4B activity in the sarcolemmal LTCC complex[16] leads to catecholamine-induced ventricular arrhythmias. In human patients with heart failure, inhibition of PDE3 by milrinone favors the development of malignant arrhythmias.[17]

Phosphoinositide 3-kinase γ (PI3Kγ) is an emerging regulator of PDE action in the myocardium. In isolated cardiomyocytes, PI3Kγ is required for activation of PDE4 in the vicinity of the SR through an as-yet unknown mechanism.[18] In addition, cardiac PI3Kγ acts as an A-kinase anchoring protein that tethers PDE3B and its activator PKA within the same macromolecular complex to enhance PDE3B activity.[19] Nonetheless, the role of PI3Kγ in arrhythmogenesis is presently unknown.

Here, we report that PI3Kγ protects against catecholamine-induced ventricular arrhythmia by linking β_2-AR signaling to PKA-mediated activation of the major PDEs controlling cardiac function, PDE4A, PDE4B and PDE3A. The resulting feedback loop limits β_2-AR–

induced cAMP elevation and PKA-dependent phosphorylation of LTCC and phospholamban, eventually preventing spontaneous arrhythmogenic Ca^{2+} release.

Methods

Expanded methods can be found in the Methods section in the online-only Data Supplement.

Mice and Surgical Procedure

PI3Kγ-deficient mice (PI3K$\gamma^{-/-}$) and knock-in mice with catalytically inactive PI3Kγ (PI3K$\gamma^{KD/KD}$) were generated as previously described.[20,21] Mutant mice were backcrossed with C57Bl/6 mice for 15 generations to inbreed the genetic background, and C57Bl/6 mice (PI3K$\gamma^{+/+}$) were used as controls. Mechanical stress was imposed on the left ventricle by transverse aortic constriction between the truncus anonymous and the left carotid artery, as previously reported.[21]

ECG Recording

For evaluation of epinephrine-induced arrhythmias, mice were anesthetized with 1% isoflurane and subjected to intraperitoneal injection of the indicated drugs under continuous ECG monitoring with a Vevo 2100 echocardiograph (VisualSonics, Toronto, Canada). In transverse aortic constriction–treated animals, serial ECG monitoring was performed 4 times daily, for a total of 4 hours, starting on day 3 after surgery.

Fluorescence Resonance Energy Transfer Imaging

Spontaneously beating neonatal cardiomyocytes were cultured on fibronectin-coated tissue culture dishes in a Dulbecco modified Eagle medium/Medium 199 (Gibco, Carlsbad, CA) mix containing 10% horse serum, 5% FBS, and 5 mmol/L penicillin/streptomycin. At 12 to 24 hours after plating, cells were infected with an adenovirus encoding Epac2-cAMPs[22] or pm-Epac2-cAMPs, a plasma membrane-targeted version of Epac2-cAMPs,[23] and live cell imaging was performed 24-hours after adenovirus infection, as previously described.[24]

Ca^{2+} Measurements

For Ca^{2+} spark measurements, adult ventricular cardiomyocytes were loaded with the Ca^{2+} fluorescence dye fluo-4AM (Molecular Probes, Invitrogen Corp, Carlsbad, CA), as previously described.[25] Ca^{2+} sparks were visualized in quiescent cardiomyocytes by a Leica SP5 confocal microscope (Leica Microsystems Inc, Germany) fitted with a white-light laser tuned to 500 nm. For Ca^{2+} transient measurements, adult ventricular cardiomyocytes were loaded with Fura-2AM (Molecular Probes) and field stimulated at a frequency of 0.5 Hz. The Fura-2 ratios were recorded with an IonOptix System (IonOptix, Milton, MA), as previously detailed.[16]

PDE Assay

PDE activity in immunoprecipitates was measured according to the 2-step method of Thompson and Appleman,[26] as previously described.[27]

Statistical Analysis

Prism software (GraphPad software Inc, La Jolla, CA) was used for statistical analysis. *P* values were calculated with the Kruskal-Wallis nonparametric test followed by the Dunn post hoc analysis. The Fisher exact test was used to evaluate arrhythmia incidence, and the log-rank test was used for survival analysis.

Results

PI3K*γ*-Null Mice Are Susceptible to β_2-AR–Triggered Ventricular Arrhythmia

To evaluate the effect of PI3Kγ on catecholamine-induced arrhythmia, ECGs were recorded in PI3K$\gamma^{+/+}$, PI3K$\gamma^{-/-}$, and PI3K$\gamma^{KD/KD}$ animals treated with epinephrine (2 mg/kg IP). Basal heart rate was similar in all genotypes, in line with previous reports.[21,28] However, the chronotropic effect of epinephrine was 10% higher in PI3K$\gamma^{-/-}$ than in PI3K$\gamma^{+/+}$ and PI3K$\gamma^{KD/KD}$ animals (Figure 1A and Table I in the online-only Data Supplement). Interestingly, epinephrine-treated PI3K$\gamma^{-/-}$ mice displayed runs of premature ventricular beats, whereas no runs were observed in PI3K$\gamma^{+/+}$ and PI3K$\gamma^{KD/KD}$ animals (Figure 1B and 1C). These data indicate that the kinase-independent function of PI3Kγ regulates both the chronotropic and arrhythmogenic effects of myocardial β-AR stimulation.

PI3Kγ is a negative regulator of β_2-AR signaling.[29,30] Furthermore, enhanced activation of cardiac β_2-ARs has been linked to the development of ventricular arrhythmias.[31,32] Accordingly, pretreatment with the selective β_2-AR antagonist ICI-118551 (2 mg/kg IP) reduced the positive chronotropic effect of epinephrine (Figure 1A) and abolished the occurrence of ventricular runs in PI3K$\gamma^{-/-}$ animals (Figure 1B and 1C and Table I in the online-only Data Supplement). These data indicate that arrhythmias occurring in PI3K$\gamma^{-/-}$ hearts are related to abnormal β_2-AR signaling. Next, to evaluate the role of PI3Kγ-related arrhythmogenesis in heart failure, mice were subjected to transverse aortic constriction, a model characterized by the endogenous adrenergic stimulation of the myocardium. Of note, transverse aortic constriction caused substantially higher mortality in PI3K$\gamma^{-/-}$ (59% on day 7) than in PI3K$\gamma^{+/+}$ (8%) and PI3K$\gamma^{KD/KD}$ (12%) mice ($P<0.01$; Figure 1D). Serial ECG monitoring of transverse aortic constriction–treated animals revealed that PI3K$\gamma^{-/-}$ mice developed sustained ventricular tachycardia immediately before death (Figure 1E).

Together, these data demonstrate that the scaffolding function of PI3Kγ protects against catecholamine-induced ventricular arrhythmia in both normal and failing hearts.

PI3K*γ* Controls β_2-AR/cAMP Responses Through Compartmentalized PDE3 and PDE4

The relation between PI3Kγ and β_2-AR/cAMP signaling was evaluated in neonatal cardiomyocytes expressing the fluorescence resonance energy transfer sensor for intracellular cAMP, Epac2-cAMPs[22] (Figure 2A, insets). Activation of β_2-ARs by short application of isoproterenol (100 nmol/L, 15 seconds) combined with the β_1-AR antagonist CGP-20712A (100 nmol/L) produced a transient increase in cAMP that returned to baseline in <5 minutes (Figure 2A). The decay of a cAMP response to a brief application of isoproterenol reflects the activity of cAMP PDEs.[24] As indicated by τ decay values, cAMP decay was 30% slower in PI3K$\gamma^{-/-}$ than in PI3K$\gamma^{+/+}$ and PI3K$\gamma^{KD/KD}$ cells (Figure 2B), demonstrating that cAMP hydrolysis by PDEs is impaired in the absence of PI3Kγ.

In adult cardiomyocytes, cAMP generated by β_2-ARs is degraded by PDE3 and PDE4.[14,15] A similar scenario was found in neonatal cells in which concomitant inhibition of PDE3 and PDE4 with Cilostamide (1 μmol/L) and Ro-201724 (10 μmol/L), respectively, almost completely blocked cAMP degradation (Figure IA–ID in the online-only Data Supplement). The selective contribution of PDE3 and PDE4 was then assessed. PDE3 inhibition by Cilostamide (1 μmol/L) significantly slowed cAMP decay in all genotypes (Figure IIA–IIC and Table II in the online-only Data Supplement), indicating that PDE3 is required to limit β_2-AR–dependent cAMP. When PDE3 is blocked by Cilostamide, the decay of cAMP reflects the activity of PDE4.[24] The finding that cAMP decay was 2-fold slower in PI3K$\gamma^{-/-}$ than in PI3K$\gamma^{+/+}$ and PI3K$\gamma^{KD/KD}$ cardiomyocytes (Figure 2C and 2D) indicated that PDE4 function is impaired in the absence of PI3Kγ. Inhibition of PDE4 by Ro-201724 (10 μmol/L)

also significantly delayed cAMP decay (Figure IIIA–IIIC and Table II in the online-only Data Supplement), demonstrating that PDE4 controls β_2-AR–dependent cAMP. When PDE4 is inhibited by Ro-201724, the rate of cAMP decay reveals the activity of PDE3.[24] In these conditions, cAMP degradation was 1.5-fold slower in PI3K$\gamma^{-/-}$ than in PI3K$\gamma^{+/+}$ and PI3K$\gamma^{KD/KD}$ cardiomyocytes (Figure 2E and 2F), thus indicating that PDE3 fails to restore basal cAMP levels in cells lacking PI3Kγ.

Because of the compartmentalization of cardiac PDEs,[33] PDE3 and PDE4 activities in the vicinity of β_2-ARs can differ from those in the bulk cytosol. Hence, subsarcolemmal β_2-AR/cAMP responses were analyzed in cardiomyocytes expressing pm-Epac2-cAMPs[23] (Figure IVA in the online-only Data Supplement, insets). β_2-AR activation increased cAMP transiently in PI3K$\gamma^{+/+}$ and PI3K$\gamma^{KD/KD}$ cardiomyocytes, whereas PI3K$\gamma^{-/-}$ responses were abnormally prolonged (Figure IVA in the online-only Data Supplement). Accordingly, half-decay time was 3-fold higher in PI3K$\gamma^{-/-}$ than in PI3K$\gamma^{+/+}$ and PI3K$\gamma^{KD/KD}$ cardiomyocytes (Figure IVB in the online-only Data Supplement). Thus, PI3Kγ limits β_2-AR–dependent cAMP near the sarcolemma. Cilostamide did not affect cAMP decay in all genotypes (Figure VA–VC and Table III in the online-only Data Supplement), indicating that PDE3 does not control subsarcolemmal cAMP. In these conditions, cAMP decay reflects PDE4 activity.[24] cAMP degradation occurred 3-fold more slowly in PI3K$\gamma^{-/-}$ than in PI3K$\gamma^{+/+}$ and PI3K$\gamma^{KD/KD}$ cardiomyocytes (Figure VD and VE in the online-only Data Supplement), thus demonstrating that PDE4 fails to terminate subsarcolemmal β_2-AR/cAMP responses in the absence of PI3Kγ. The finding that Ro-201724 almost completely blocked cAMP hydrolysis in all genotypes (Figure VIA–VIE and Table III in the online-only Data Supplement) confirmed that PDE4 limits mainly subsarcolemmal cAMP. These data indicate that PI3Kγ controls PDE3 and PDE4 in distinct subcellular compartments.

To further prove a major involvement of PI3Kγ scaffold function, β_2-AR/cAMP responses were measured by a ICUE3 probe[34] in cardiomyocytes expressing a kinase-dead mutant PI3Kγ (PI3KγKD-RFP; Figure VIIA in the online-only Data Supplement). In PI3K$\gamma^{-/-}$ neonatal cardiomyocytes, transfected PI3KγKD-RFP fully rescued β_2-AR/cAMP responses to the levels of PI3K$\gamma^{+/+}$ cells (Figure VIIB and VIIC in the online-only Data Supplement).

Together, these data unveil a critical role for the scaffold function of PI3Kγ in terminating β_2-AR/cAMP signaling via compartmentalized modulation of PDE3 and PDE4.

PI3K*γ* Activates PDE4A, PDE4B, and PDE3A via PKA

Different PDE3 and PDE4 isoenzymes are expressed in the myocardium.[13] The specific isoforms regulated by PI3Kγ were thus analyzed in adult whole hearts. The catalytic activity of PDE4A and PDE4B was 20% lower in PI3K$\gamma^{-/-}$ than in PI3K$\gamma^{+/+}$ and PI3K$\gamma^{KD/KD}$ heart membranes (Figure 3A and 3B) but was unchanged in cytosolic fractions and total lysates (Figure VIIIB in the online-only Data Supplement). Conversely, the activity of PDE4D, the other major myocardial PDE4 isoform, was independent of PI3Kγ (Figure IXA in the online-only Data Supplement). In addition to PDE3B,[21] PDE3A activity was found to be 30% lower in PI3K$\gamma^{-/-}$ than in PI3K$\gamma^{+/+}$ and PI3K$\gamma^{KD/KD}$ heart membranes (Figure 3C) but not in cytosolic fractions and total lysates (Figure VIIIC in the online-only Data Supplement). Thus, PI3Kγ regulates membrane-bound PDE4A, PDE4B, and PDE3A but not PDE4D.

The reduction of PDE activities detected in PI3K$\gamma^{-/-}$ membranes was not linked to a decreased amount of PDE enzymes in this compartment (Figure XA–XC in the online-only Data Supplement). Thus, PI3Kγ might promote PDE activation through a protein-protein interaction mechanism. Consistently, PI3Kγ copurified with the long 95-kDa isoform of PDE4A and with the long 92-kDa variant of PDE4B in adult hearts (Figure 3D and 3E).

Two distinct PDE3A isoforms of 97 and 106 kDa also coprecipitated with PI3Kγ (Figure 3F). In line with cAMP PDE measurements, PI3Kγ was not found to interact with PDE4D (Figure IXB in the online-only Data Supplement). These data indicate that PI3Kγ physically associates with and modulates PDE4A, PDE4B, and PDE3A but not PDE4D.

PI3Kγ-associated PDE3B is activated by anchored PKA.[19] Because PKA also activates PDE3A[35] and long PDE4 isoforms,[36] the ability of PI3Kγ to operate PKA-mediated activation of other PDEs was investigated. Of note, PDE4A, PDE4B, and PDE3A were part of macromolecular complexes containing PI3Kγ together with the regulatory and catalytic subunits of PKA (Figure 4A–4C). In isolated cardiomyocytes, the PKA inhibitor Myr-PKI (5 μmol/L, 10 minutes) abolished the PI3Kγ-dependent increase in PDE4A, PDE4B, and PDE3A activity (Figure 4D–4F). To further support the involvement of PKA, interaction studies in HEK293 cells expressing either a wild-type PI3Kγ (PI3KγWT) or a mutant PI3Kγ that cannot bind PKA (PI3KγK126A,R130A)[19] were performed. Transfected PI3KγWT copurified with the long PDE4A variant endogenously expressed by HEK293 cells and increased PDE4A-mediated hydrolysis of cAMP by 30% (Figure 5A). On the contrary, PI3KγK126A,R130A failed to enhance PDE4A activity while retaining the ability to copurify with the enzyme (Figure 5A). Similarly, the catalytic activity of transfected PDE4B and PDE3A was significantly increased by the association with PI3KγWT but not with PI3KγK126A,R130A (Figure 5B and 5C). Thus, a loss of PKA anchoring prevents PI3Kγ-dependent enhancement of PDE4A, PDE4B, and PDE3A activity.

Together, these data indicate that PI3Kγ is a multifunctional A-kinase anchoring protein that limits β_2-AR/cAMP responses via PKA-mediated activation of different PDEs.

cAMP-Mediated Phosphorylation of $Ca_v1.2$ and Phospholamban Is Increased in PI3Kγ-Null Cardiomyocytes

The impact of PI3Kγ on cAMP-mediated signal transduction was evaluated next. In cardiomyocytes, cAMP-activated PKA modulates crucial effectors of excitation-contraction coupling such as LTCC, RyR, phospholamban, and troponin I.[4] PKA-mediated phosphorylation of the LTCC pore-forming subunit $Ca_v1.2$ was 3-fold higher in PI3K$\gamma^{-/-}$ than in PI3K$\gamma^{+/+}$ cardiomyocytes after β_2-AR activation (Figure 6A). Consistent with a major role of PI3Kγ in controlling sarcolemmal PDE4, $Ca_v1.2$ phosphorylation was significantly enhanced in PI3K$\gamma^{-/-}$ over PI3K$\gamma^{+/+}$ cardiomyocytes when the contribution of PDE4 was revealed by Cilostamide (Figure XI in the online-only Data Supplement). Moreover, PI3Kγ was found to be physically associated with $Ca_v1.2$ (Figure 6B), further supporting the view that PI3Kγ limits β_2-AR/cAMP signaling at the sarcolemma in proximity of the LTCC.

At the SR, PKA phosphorylates RyR and phospholamban.[4] Ser-2808 RyR phosphorylation was unchanged in PI3K$\gamma^{-/-}$ compared with PI3K$\gamma^{+/+}$ cardiomyocytes after β_2-AR stimulation (Figure 6C). On the contrary, Ser-16 phospholamban phosphorylation was 2.3-fold higher in PI3K$\gamma^{-/-}$ than in PI3K$\gamma^{+/+}$ cardiomyocytes (Figure 6D). In addition, phospholamban phosphorylation was significantly higher in PI3K$\gamma^{-/-}$ than in PI3K$\gamma^{+/+}$ cells when either PDE3 or PDE4 was inhibited by Cilostamide or Ro-201724 (Figure XIIA and XIIB in the online-only Data Supplement). These findings indicate that PI3Kγ-activated PDE3 and PDE4 delimit β_2-AR/cAMP signaling at the SR in proximity of phospholamban but not of RyR. Similar to phospholamban, another intracellular target of PKA, troponin I, was hyperphosphorylated in PI3K$\gamma^{-/-}$ cardiomyocytes on β_2-AR activation and when PDE3 and PDE4 were selectively blocked (Figure XIIIA–XIIIC).

Together, these data indicate that PI3Kγ affects key regulators of ventricular cardiomyocyte excitability by controlling local pools of β_2-AR/cAMP.

PI3K*γ*-Null Cardiomyocytes Develop Increased Spontaneous Ca^{2+} Release Events

cAMP-mediated phosphorylation of $Ca_v1.2$ and phospholamban enhances LTCC current amplitude and accelerates SR Ca^{2+} reuptake, respectively.[4] Previous evidence demonstrated that PI3K$\gamma^{-/-}$ adult cardiomyocytes have higher LTCC current density than PI3K$\gamma^{+/+}$ cells after β_2-AR activation.[30] To explore the role of PI3Kγ in Ca^{2+} homeostasis further, SR Ca^{2+} release was analyzed in quiescent and epinephrine-treated adult cardiomyocytes (Figure 7A). Ca^{2+} spark frequency was not significantly different between PI3K$\gamma^{+/+}$ and PI3K$\gamma^{-/-}$ cells after epinephrine (Figure 7B). In contrast, the effect of epinephrine on Ca^{2+} spark occurrence was higher in PI3K$\gamma^{-/-}$ than in PI3K$\gamma^{+/+}$ cardiomyocytes (Figure 7C), revealing a hyperresponsiveness of PI3K$\gamma^{-/-}$ cells to adrenergic stimulation. In addition, Ca^{2+} spark amplitude was significantly increased in PI3K$\gamma^{-/-}$ cardiomyocytes in basal conditions and further enhanced by adrenergic stimulation (Figure 7D). Thus, spontaneous SR Ca^{2+} release via RyR is enhanced in the absence of PI3Kγ.

The impact of local Ca^{2+} mishandlings on global intracellular Ca^{2+} was evaluated next. Intracellular Ca^{2+} transients were recorded in electrically paced (0.5 Hz) adult cardiomyocytes after application of epinephrine alone (100 nmol/L) or in combination with ICI-118551 (100 nmol/L). Spontaneous Ca^{2+} release events were more frequent in PI3K$\gamma^{-/-}$ than in PI3K$\gamma^{+/+}$ and PI3K$\gamma^{KD/KD}$ cardiomyocytes (Figure 8A–8C). In line with in vivo experiments (Figure 1), ICI-118551 abolished the occurrence of arrhythmic spontaneous Ca^{2+} release events induced by epinephrine in PI3K$\gamma^{-/-}$ cells (Figure 8A–8C). Thus, the scaffold function of PI3Kγ prevents spontaneous Ca^{2+} release events after activation of β_2-ARs.

Together, these data demonstrate that PI3Kγ limits β_2-AR–dependent arrhythmogenic Ca^{2+} release via PKA-mediated activation of PDE4A, PDE4B, and PDE3A.

Discussion

The present study unravels a major role of PI3Kγ in the protection against catecholamine-induced ventricular arrhythmia. PI3K$\gamma^{-/-}$ mice developed runs of premature ventricular contractions on β-AR stimulation caused by aberrant Ca^{2+} release in ventricular cardiomyocytes. This proarrhythmic phenotype stems from a functional impairment in multiple cAMP PDEs, which leads to uncontrolled cAMP/PKA signaling. Our findings picture a scenario in which PI3Kγ orchestrates multi-protein complexes controlling both PKA-mediated activation of PDEs (PDE3A, PDE4A, PDE4B) and a physiological feedback inhibition of the $Ca_v1.2$ LTCC subunit and phospholamban.

The full rescue of ventricular arrhythmia with the β_2-AR antagonist ICI-118551 indicates a selective engagement of PI3Kγ downstream from the β_2-AR subtype. This finding is in agreement with the previous report of the increased cAMP accumulation detected in PI3K$\gamma^{-/-}$ cardiomyocytes after stimulation with the β_2-AR agonist zinterol.[29] Furthermore, these results are consistent with evidence that the β_2-AR represents de facto the major β-AR isoform involved in arrhythmogenesis.[31,32] Although our measurements excluded supraventricular arrhythmias, epinephrine-induced sinus tachycardia was more pronounced in PI3K$\gamma^{-/-}$ than in PI3K$\gamma^{+/+}$ and PI3K$\gamma^{KD/KD}$ animals. This finding implies that PI3Kγ also influences sinoatrial node function in vivo and supports previous evidence that PI3Kγ increases spontaneous pacemaker activity in isolated sinoatrial node myocytes.[28]

It has previously been reported that PI3Kγ directly associates with PKA and acts as an A-kinase anchoring protein involved in the negative regulation of cardiac cAMP.[19] The present study further demonstrates that PI3Kγ orchestrates the activity of multiple PDEs, including those with a major impact on cardiac function such as PDE4A, PDE4B, and PDE3A. This

control is independent of PI3Kγ kinase activity and depends on protein scaffolding. Whether PI3Kγ regulates PDE3 or PDE4 has been a subject of debate. In whole hearts, PI3Kγ has been shown to regulate mainly PDE3B, independently of its kinase activity.[21] In contrast, in isolated cardiomyocytes, PI3Kγ appears to modulate PDE4 but not PDE3 activity.[18] The present study provides a solution to this controversy in that PI3Kγ was found to cooperate with either PDE3 or PDE4, depending on the subcellular compartment. Fluorescence resonance energy transfer–based assays demonstrated that the main PDE activated by PI3Kγ in the cytosol is PDE3. Conversely, PI3Kγ controls PDE4- but not PDE3-dependent cAMP pools close to the plasma membrane.

The finding that PDE3 is not required for the modulation of β_2-AR/cAMP signaling at the plasma membrane was unexpected because PDE3 isoenzymes are known to be membrane bound.[37–39] However, PDE3 localizes mainly at the SR/endoplasmic reticulum rather than at the plasma membrane.[38] In agreement with this idea, PDE3A was modulated by PI3Kγ in total heart membranes, which contain also SR/endoplasmic reticulum membranes, but not at the plasma membrane, as detected by the pm-Epac2-cAMPs sensor. On the other hand, the major role of PDE4 in controlling sarcolemmal cAMP is supported by previous evidence that both PDE4A and PDE4B can localize to this compartment.[16,40,41] Our findings demonstrate that the activity of these pools of PDE4A and PDE4B relies on PI3Kγ scaffold activity. Taken together, present and previous data indicate that PI3Kγ regulates the coincident signaling of PDE3 and PDE4 by acting in spatially confined compartments of cardiomyocytes.

PI3Kγ-dependent tuning of multiple PDEs is required to limit PKA-mediated activation of the excitation-contraction coupling machinery, including the sarcolemmal LTCC and phospholamban at the SR. The present work demonstrates that PI3Kγ operates a feedback loop inhibiting PKA-mediated phosphorylation of the $Ca_v1.2$ subunit of LTCC on β-AR stimulation. This mechanism eventually explains the previous report of increased LTCC current density ($I_{Ca,L}$) in PI3K$\gamma^{-/-}$ cardiomyocytes[30] and the present finding that PI3K$\gamma^{-/-}$ mice develop calcium-dependent arrhythmia. Similarly, enhanced activation of LTCC is the main trigger of ventricular tachycardia detected in PDE4B$^{-/-}$ mice.[16] The proarrhythmic effect of uncontrolled LTCC function has also been shown in humans, in whom a missense mutation of $Ca_v1.2$ causing increased channel opening leads to severe arrhythmias.[42] PDE4B is part of the $Ca_v1.2$ channel complex and acts as a negative regulator of $I_{Ca,L}$ under β-AR stimulation.[16] The finding that PI3Kγ associates with $Ca_v1.2$ suggests that PI3Kγ-mediated control of PDE4B activity in the $Ca_v1.2$ channel complex is another mechanism by which PI3Kγ confers protection against cardiac arrhythmia.

The effects of enhanced $I_{Ca,L}$ detected in PI3K$\gamma^{-/-}$ cardiomyocytes can be further strengthened by increased diastolic Ca^{2+} release caused by more intense Ca^{2+} sparks. This effect can be indirectly linked to enhanced Ca^{2+} entry through hyperphosphorylated LTCC and to hyperphosphorylation of phospholamban, which in turn stimulates Ca^{2+} reuptake, increasing SR Ca^{2+} load. On the contrary, PKA-mediated phosphorylation of RyR did not require PI3Kγ, and Ca^{2+} spark frequency was thus maintained in PI3K$\gamma^{-/-}$ cardiomyocytes. This is consistent with previous evidence that RyR activity is PI3Kγ independent[18] but relies on the regulation of a complex containing PDE4D.[2] Accordingly, PI3Kγ neither associated with nor controlled the catalytic activity of PDE4D.

Conclusions

This study identifies PI3Kγ as a central switch of cAMP compartmentalization that affects multiple β_2-AR/cAMP microdomains via localized PKA-mediated activation of distinct PDEs. Such spatiotemporal organization of cAMP signaling allows the physiological

regulation of cardiac function, translating β_2-AR stimulation into the appropriate cardiac response. This mechanism appears relevant to heart failure, in which ventricular arrhythmia is a major cause of death. Interestingly, failing hearts show a functional decay in PI3Kγ-directed protein complexes.[19] Hence, deregulation of PI3Kγ scaffold function may constitute an important component of heart failure-related arrhythmias.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Clinical Perspective

Ventricular arrhythmia is a leading cause of sudden death. Malignant ventricular arrhythmias such as ventricular tachycardia can develop in otherwise healthy individuals carrying proarrhythmic mutations and in patients affected by cardiomyopathies such as ischemic heart disease and heart failure. Although the administration of classic antiarrhythmic drugs (ie, β-blockers and amiodarone) and the implantation of cardiac defibrillators constitute a cornerstone of current patient management, only a better understanding of the molecular circuitries underlying ventricular arrhythmogenesis will pave our way toward new frontiers in sudden cardiac death prevention. For this purpose, experimental dissection of the molecular pathways that fine-tune both second messenger signaling and excitation-contraction coupling in cardiomyocytes is paramount. In the present work, using genetically modified mice, we have uncovered that the enzyme phosphoinositide 3-kinase γ (PI3Kγ) is required to maintain a physiological function of important myocardial phosphodiesterases such as phosphodiesterase 3A, 4A, and 4B. Biochemical and functional data indicate that PI3Kγ constitutes a necessary scaffold for these phosphodiesterases because the loss of PI3Kγ leads to abnormal cAMP accumulation, to inappropriate activation of cAMP targets such as L-type calcium channel, and to spontaneous calcium release events in cardiomyocytes. Although in normal hearts the loss of PI3Kγ leads to benign premature ventricular beats on β-adrenergic stimulation, cardiac pressure overload precipitates the development of ventricular tachycardia and rapidly results in substantial mortality. These findings indicate that the β-adrenergic/PI3Kγ/phosphodiesterase signaling hub may constitute a promising molecular target for the development of novel antiarrhythmic therapeutic interventions.

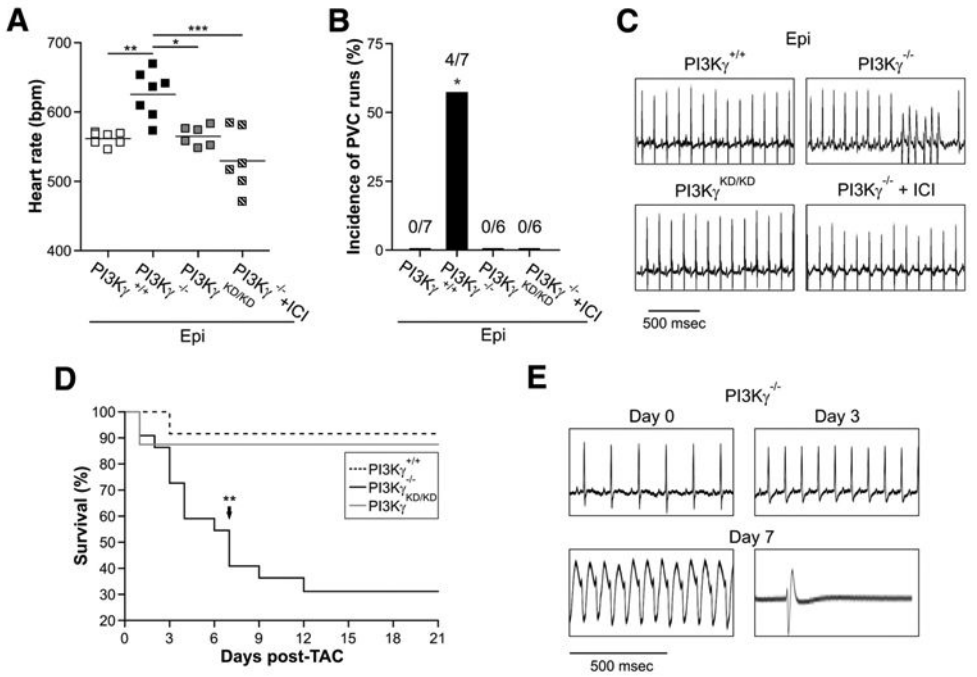

Figure 1.

Phosphoinositide 3-kinase γ (PI3Kγ) protects against β_2-adrenergic receptor (AR)–induced ventricular arrhythmia. **A**, Heart rate of PI3K$\gamma^{+/+}$ (n=7), PI3K$\gamma^{-/-}$ (n=7), and PI3K$\gamma^{KD/KD}$ (n=6) mice after the indicated treatments. ECG was obtained 30 minutes after injection of epinephrine (Epi; 2 mg/kg IP) or Epi plus the selective β_2-AR antagonist ICI-118551 (ICI; 2 mg/kg IP). *P<0.05, **P<0.01, ***P<0.001. **B**, Incidence of premature ventricular contraction (PVC) runs (percent of treated mice) after treatment with Epi or Epi+ICI. The number of mice developing PVC runs over the number of total mice per group is reported above each bar graph. *P<0.05 by the Fisher exact test. **C**, Representative ECG traces of PI3K$\gamma^{+/+}$, PI3K$\gamma^{-/-}$, and PI3K$\gamma^{KD/KD}$ mice recorded 30 minutes after injection of Epi or Epi +ICI. **D**, Kaplan-Meier survival curve of PI3K$\gamma^{+/+}$ (n=12), PI3K$\gamma^{-/-}$ (n=21), and PI3K$\gamma^{KD/KD}$ (n=8) mice 3 weeks after transverse aortic constriction (TAC). **P<0.01 by log-rank test. **E**, Representative ECG traces of a PI3K$\gamma^{-/-}$ mouse on day 0 (immediately before TAC), day 3 after TAC, and day 7 after TAC. Day 0, normal sinus rhythm; day 3, sinus tachycardia; day 7, sustained ventricular tachycardia leading to asystole.

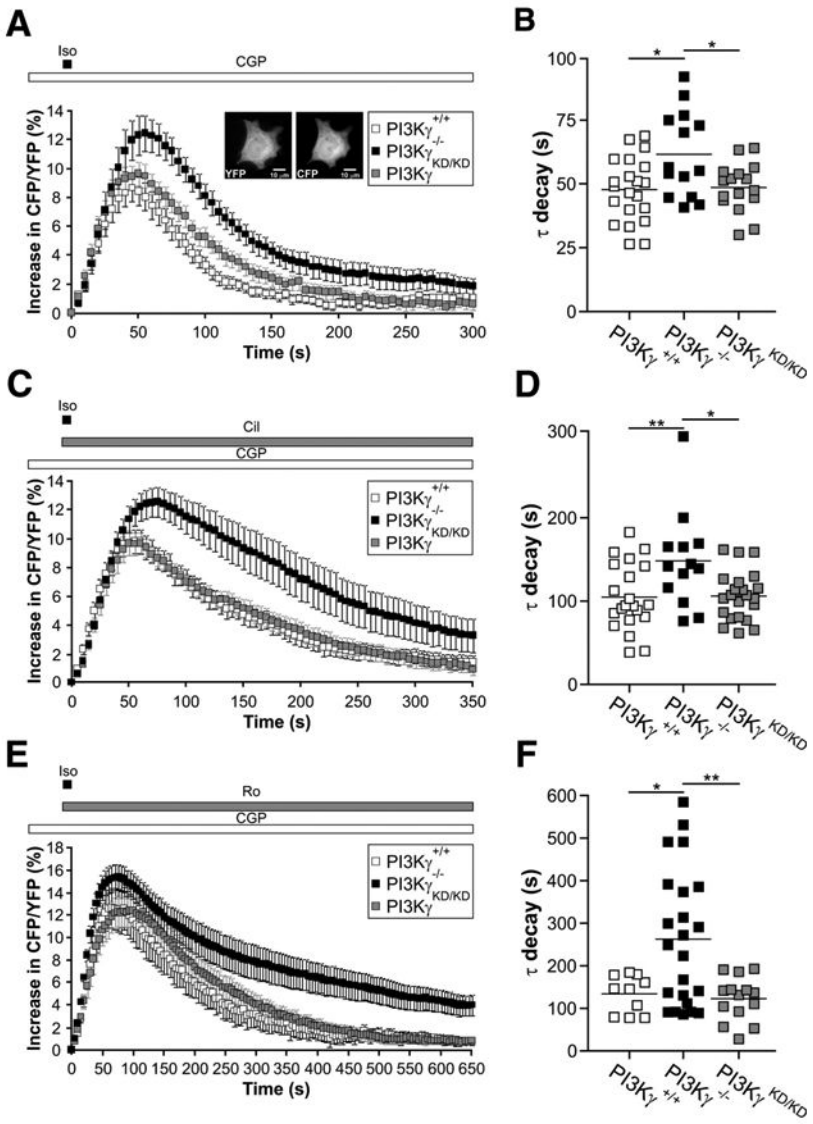

Figure 2.

Phosphoinositide 3-kinase γ (PI3Kγ) limits β_2- adrenergic receptor (AR)/cAMP transients via compartmentalized phosphodiesterase (PDE) 3 and PDE4. **A**, A fluorescence resonance energy transfer (FRET)–based sensor for cytosolic cAMP (Epac2-cAMPs) was expressed in cardiomyocytes, and β_2-ARs were selectively activated by short application of isoproterenol (Iso; 100 nmol/L, 15 seconds) in the presence of the β_1-AR selective antagonist CGP-20712A (CGP; 100 nmol/L). FRET traces from PI3K$\gamma^{+/+}$ (n=22), PI3K$\gamma^{-/-}$ (n=16), and PI3K$\gamma^{KD/KD}$ (n=18) neonatal cardiomyocytes are presented. Insets, Representative cyan and yellow fluorescence protein images. **B**, Decay kinetics (τ decay) of cAMP responses shown in **A. C**, FRET traces obtained from PI3K$\gamma^{+/+}$ (n=21), PI3K$\gamma^{-/-}$ (n=16), and PI3K$\gamma^{KD/KD}$ (n=24) neonatal cardiomyocytes treated with Iso, CGP, and the selective PDE3 inhibitor Cilostamide (Cil; 1 μmol/L). **D**, Decay kinetics (τ decay) of cAMP responses shown in **C. E**, FRET traces obtained from PI3K$\gamma^{+/+}$ (n=12), PI3K$\gamma^{-/-}$ (n=27), and PI3K$\gamma^{KD/KD}$ (n=15) neonatal cardiomyocytes treated with Iso, CGP, and the selective PDE4 inhibitor Ro-201724 (Ro; 10 μmol/L). **F**, Decay kinetics (τ decay) of cAMP responses shown in **E**. In **A, C**, and **E**, error bars indicate SEM. **P*<0.05, ***P*<0.01.

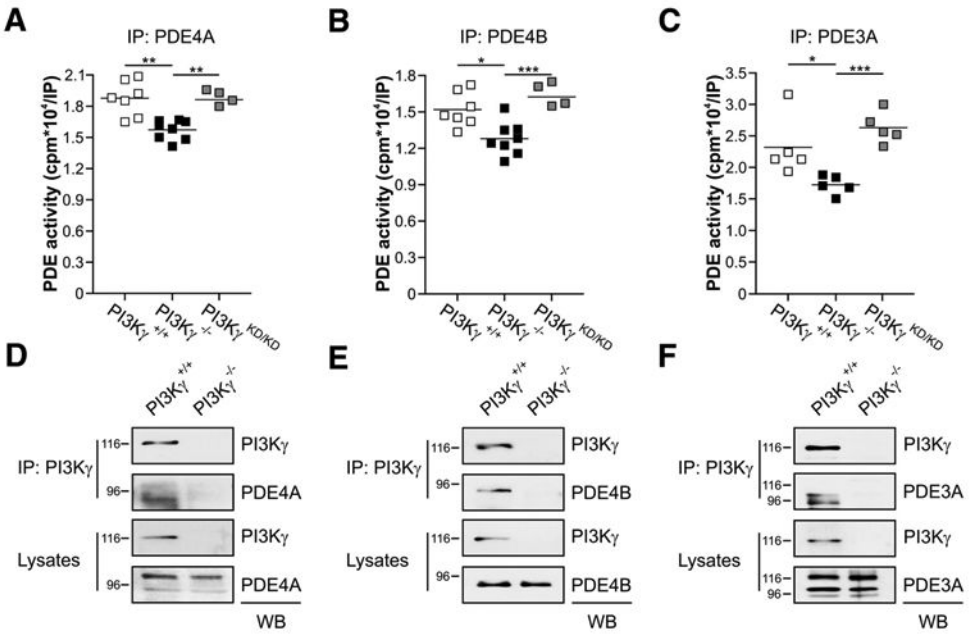

Figure 3.

Phosphoinositide 3-kinase γ (PI3Kγ) binds and modulates phosphodiesterase (PDE) 4A, PDE4B, and PDE3A. **A** through **C**, cAMP PDE activity precipitated with selective anti-PDE4A (**A**), anti-PDE4B (**B**), and anti-PDE3A (**C**) antibodies from membrane fractions of PI3K$\gamma^{+/+}$, PI3K$\gamma^{-/-}$, and PI3K$\gamma^{KD/KD}$ adult hearts (n≥4 independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001. **D** through **F**, Western blot detection of PDE4A (**D**), PDE4B (**E**), and PDE3A (**F**) in PI3Kγ immunoprecipitates from PI3K$\gamma^{+/+}$ and PI3K$\gamma^{-/-}$ heart lysates. A representative coimmunoprecipitation assay of 4 is shown.

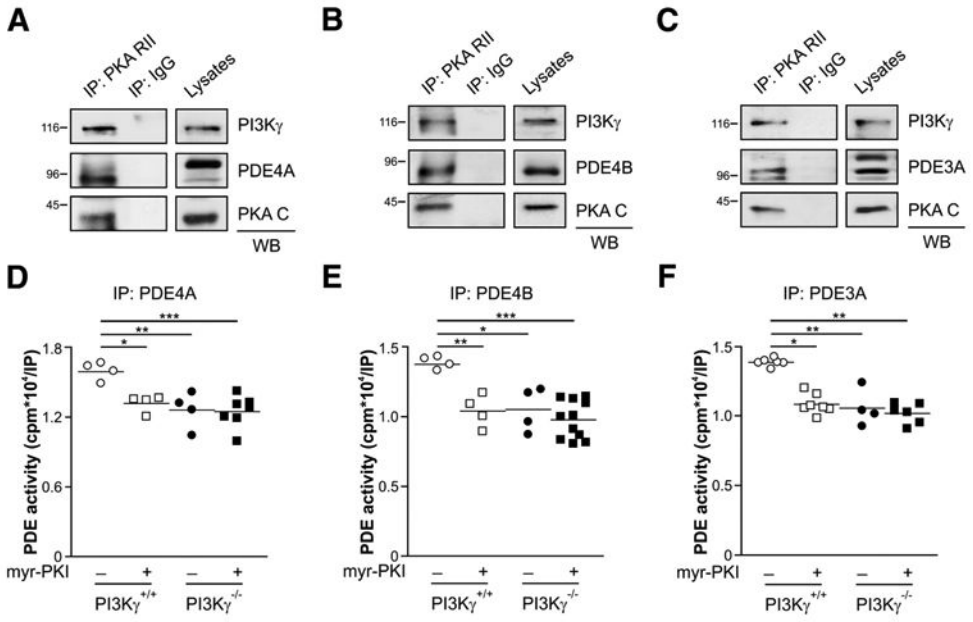

Figure 4.

Phosphoinositide 3-kinase γ (PI3Kγ) activates phosphodiesterase (PDE) 4A, PDE4B, and PDE3A via protein kinase A (PKA). **A** through **C**, Western blot detection of PDE4A (**A**), PDE4B (**B**), and PDE3A (**C**), together with PI3Kγ and PKA catalytic subunit (PKA C), in PKA regulatory subunit (PKA RII) immunoprecipitates (IPs) from PI3K$\gamma^{+/+}$ hearts. A representative experiment of 4 is shown. **D** through **F**, cAMP PDE activity precipitated by anti-PDE4A (**D**), anti-PDE4B (**E**), and anti-PDE3A (**F**) antibodies from PI3K$\gamma^{+/+}$ and PI3K$\gamma^{-/-}$ neonatal cardiomyocytes treated with either vehicle or the PKA inhibitor Myr-PKI (5 μmol/L, 10 minutes; n≥4 independent experiments). *P<0.05, **P<0.01, ***P<0.001.

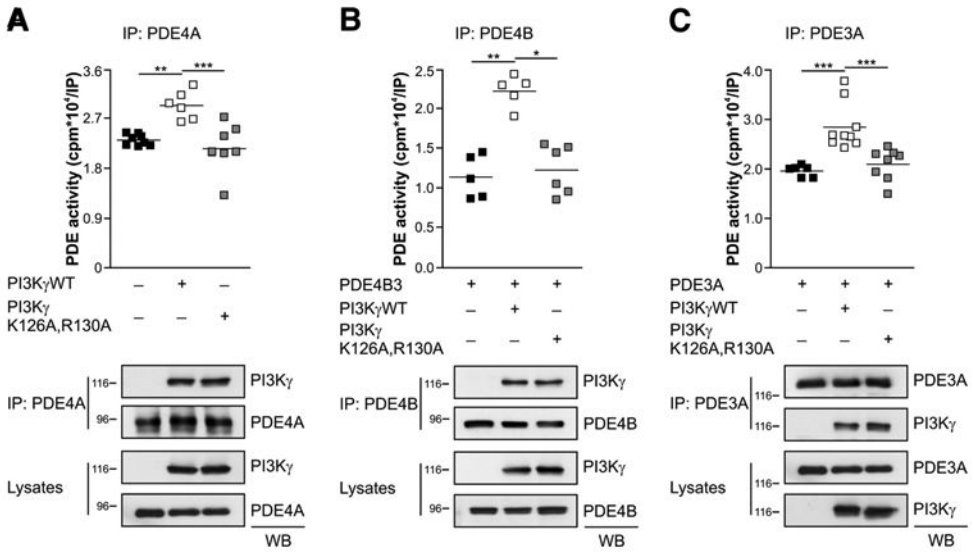

Figure 5.

A protein kinase A (PKA)–anchoring defective phosphoinositide 3-kinase γ (PI3Kγ) fails to activate phosphodiesterase (PDE) 4A, PDE4B, and PDE3A. **A** through **C**, cAMP PDE activity of endogenous PDE4A (**A**), transfected PDE4B (**B**), and transfected PDE3A (**C**) in HEK293 cells overexpressing either wild-type PI3Kγ (PI3KγWT) or a mutant PI3Kγ unable to bind the PKA regulatory subunit (PI3KγK126A,R130A; n≥5 independent experiments). Representative immunoprecipitations (IPs) are provided. **P*<0.05, ***P*<0.01, ****P*<0.001.

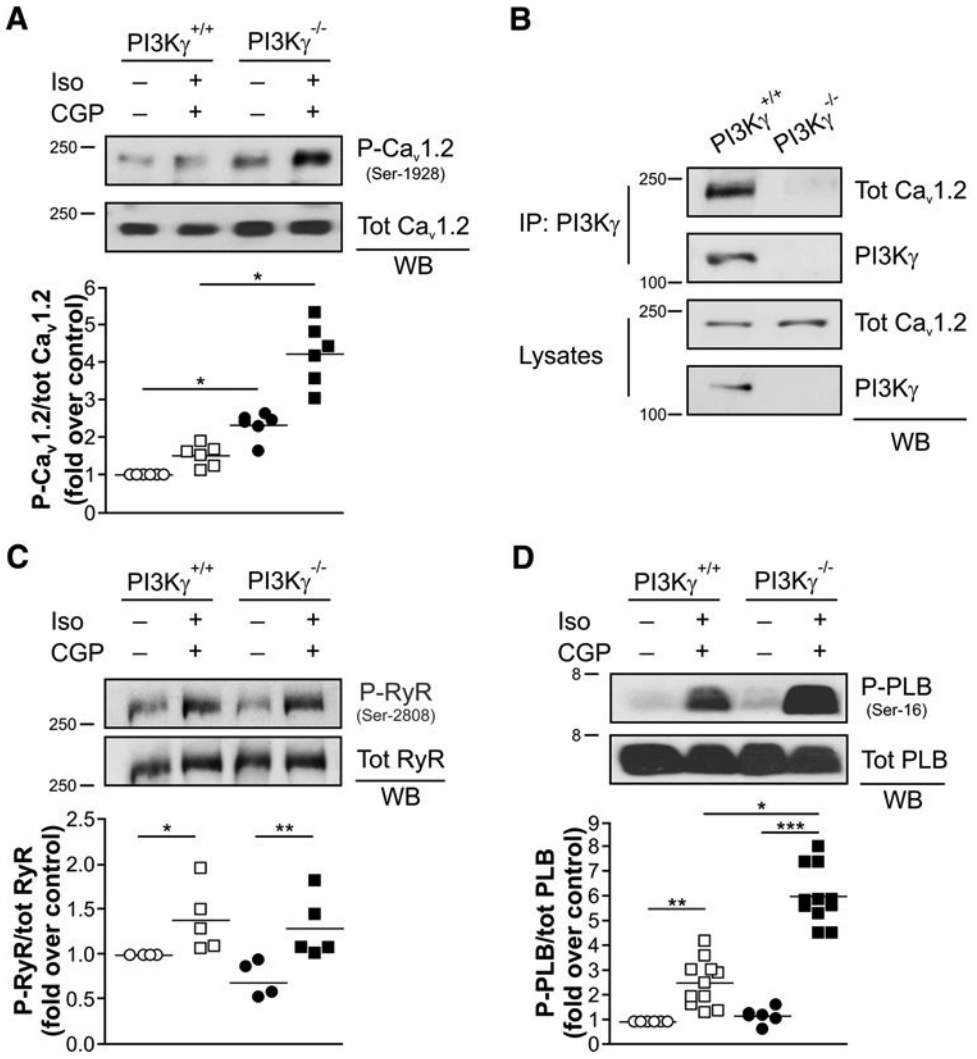

Figure 6.

cAMP-dependent phosphorylation of $Ca_v1.2$ and phospholamban (PLB) is enhanced in phosphoinositide 3-kinase γ (PI3Kγ)–null (PI3K$\gamma^{-/-}$) cardiomyocytes. **A**, Protein kinase A (PKA)–mediated phosphorylation of $Ca_v1.2$ in PI3K$\gamma^{+/+}$ and PI3K$\gamma^{-/-}$ neonatal cardiomyocytes treated with either vehicle or isoproterenol (Iso; 100 nmol/L) plus the β_1-adrenergic receptor selective antagonist CGP-20712A (CGP; 100 nmol/L) for 3 minutes. **B**, Western blot detection of $Ca_v1.2$ in PI3Kγ immunoprecipitates from PI3K$\gamma^{+/+}$ and PI3K$\gamma^{-/-}$ hearts. **C**, PKA-mediated phosphorylation of RyR in PI3K$\gamma^{+/+}$ and PI3K$\gamma^{-/-}$ cardiomyocytes treated as in **A. D**, PKA-mediated phosphorylation of PLB in PI3K$\gamma^{+/+}$ and PI3K$\gamma^{-/-}$ cardiomyocytes treated as in **A** (n≥4 independent experiments). Representative blots are provided. IP indicates immunoprecipitation. **P*<0.05, ***P*<0.01, ****P*<0.001.

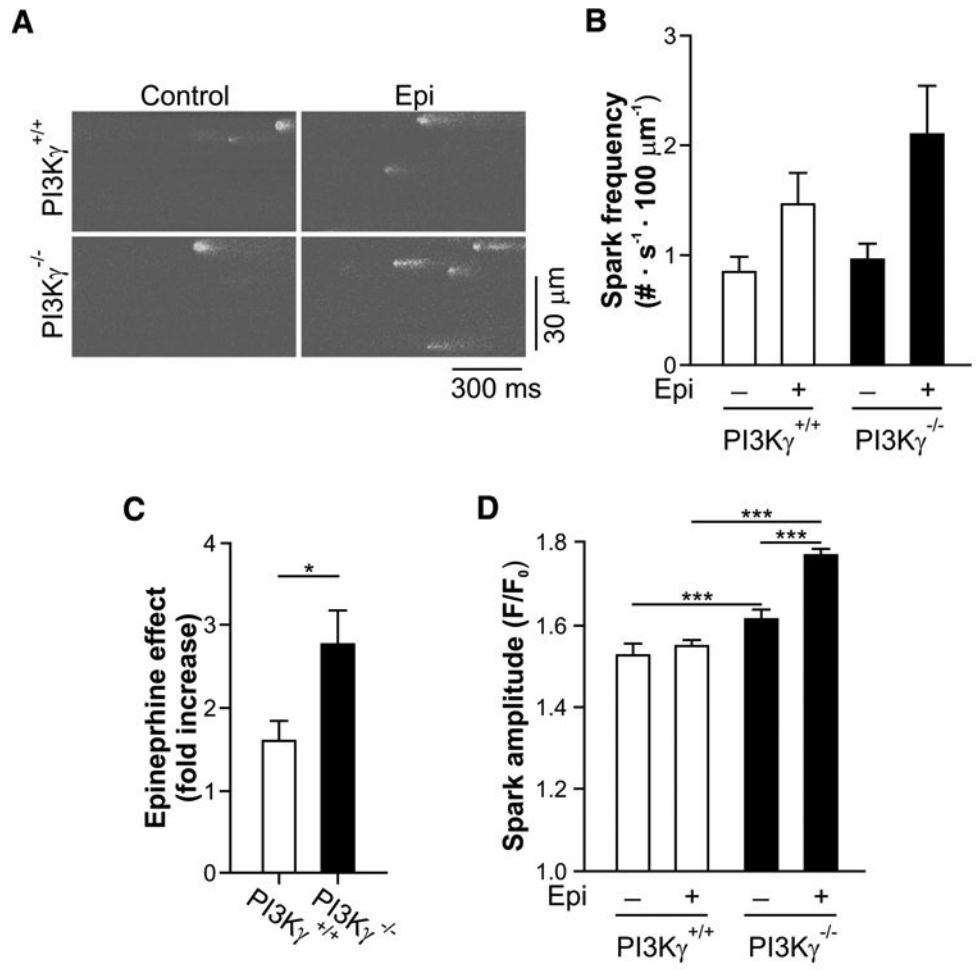

Figure 7.

Sarcoplasmic reticulum Ca^{2+} release is enhanced in phosphoinositide 3-kinase γ (PI3Kγ)–null (PI3K$\gamma^{-/-}$) cardiomyocytes. **A**, Representative line scan images of PI3K$\gamma^{+/+}$ and PI3K$\gamma^{-/-}$ adult cardiomyocytes before and during epinephrine stimulation (Epi; 1 μmol/L). **B**, Ca^{2+} spark frequency in PI3K$\gamma^{+/+}$ (n=13) and PI3K$\gamma^{-/-}$ (n=18) cardiomyocytes before and during Epi stimulation. **C**, Fold increase in Ca^{2+} spark frequency in PI3K$\gamma^{+/+}$ and PI3K$\gamma^{-/-}$ cardiomyocytes on Epi stimulation. **D**, Amplitude of Ca^{2+} sparks detected in PI3K$\gamma^{+/+}$ and PI3K$\gamma^{-/-}$ before (PI3K$\gamma^{+/+}$, n=281; PI3K$\gamma^{-/-}$, n=353) and during (PI3K$\gamma^{+/+}$, n=658; PI3K$\gamma^{-/-}$, n=1008) Epi stimulation. Error bars indicate SEM. **P*<0.05, ****P*<0.001.

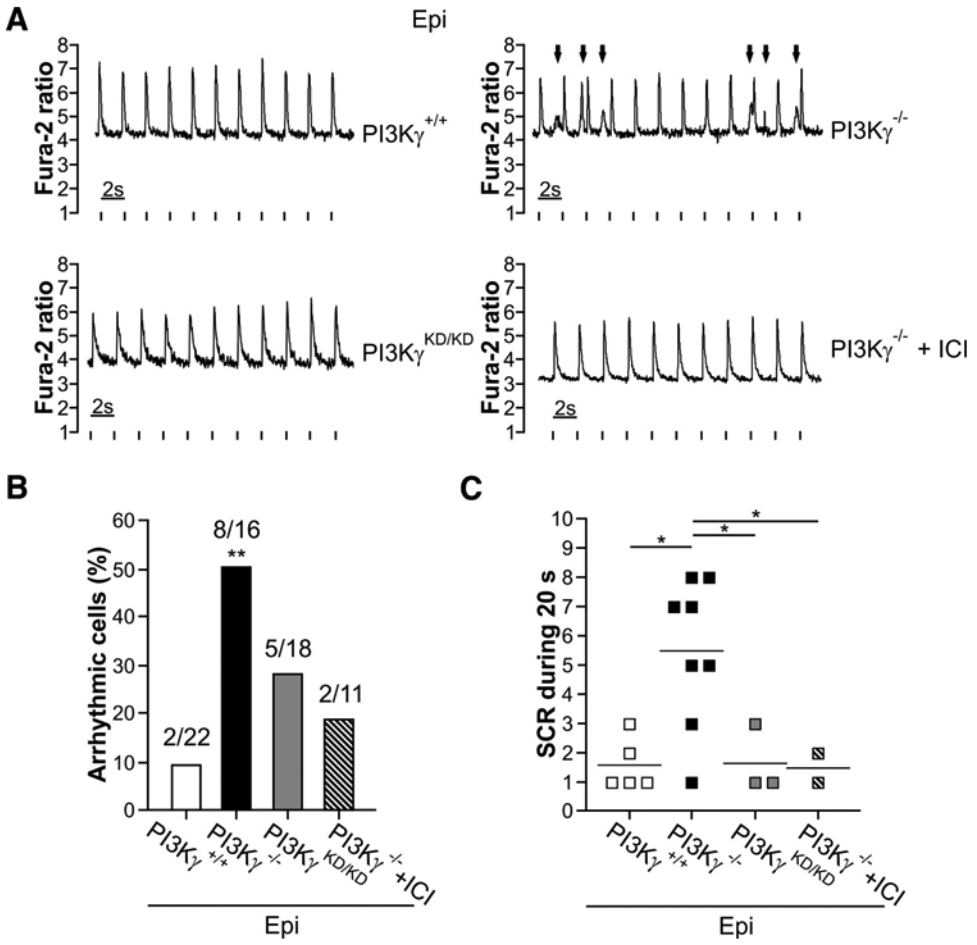

Figure 8.

Spontaneous Ca^{2+} release events are increased in phosphoinositide 3-kinase γ (PI3Kγ)–null (PI3K$\gamma^{-/-}$) cardiomyocytes. **A**, Representative traces of Ca^{2+} transients recorded in electrically paced (0.5 Hz) adult cardiomyocytes during stimulation with epinephrine (Epi; 100 nmol/L) or Epi plus the β_2-adrenergic receptor antagonist ICI-118551 (ICI; 100 nmol/L). Arrows indicate spontaneous calcium release (SCR) events. **B**, Percentage of arrhythmic cardiomyocytes during a 3-minute stimulation with Epi or Epi + ICI. The number of cardiomyocytes developing SCR events over the number of total cell per group is reported above each bar graph. $^{**}P<0.01$ by the Fisher exact test. **C**, Number of SCR events occurring in 20 seconds of stimulation with Epi or Epi+ICI application. $^{*}P<0.05$.