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

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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 (PI3Kγ−/−) showed runs of premature ventricular contractions on adrenergic stimulation that could be rescued by a selective β2-adrenergic receptor blocker and developed sustained ventricular tachycardia after transverse aortic constriction. Consistently, fluorescence resonance energy transfer probes revealed abnormal cAMP accumulation after β2-adrenergic receptor activation in PI3Kγ−/− 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 L-type calcium channel (Ca1.2) and phospholamban. In PI3Kγ−/− cardiomyocytes, Ca1.2 and phospholamban were hyperphosphorylated, leading to increased Ca2+ spark occurrence and amplitude on adrenergic stimulation. Furthermore, PI3Kγ−/− cardiomyocytes showed spontaneous Ca2+ release events and developed arrhythmia 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. (Circulation. 2012;126:2073-2083.)

Key Words: 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 Gαs, which promotes adenylyl cyclase activity and cAMP production.4 In turn, cAMP-mediated activation of PKA evokes phosphor-
ylation 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.\(^9\)

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The spatial and temporal compartmentalization of cAMP ensures that PKA encounters its substrates in the right place and at the right time.\(^6\) 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\(_2\)-ARs are much more efficient in enhancing cardiac contractility than β\(_1\)-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 enhanced cardiac contractility than β\(_2\)-ARs.\(^8\) This is due in part to the development of malignant arrhythmias.\(^17\)

Phosphoinositide 3-kinase γ (PI3K\(_γ\)) is an emerging regulator of PDE action in the myocardium. In isolated cardiomyocytes, PI3K\(_γ\) is required for activation of PDE4 in the vicinity of the SR through an as-yet unknown mechanism.\(^18\) In addition, cardiac PI3K\(_γ\) 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 PI3K\(_γ\) in arrhythmogenesis is presently unknown.

Here, we report that PI3K\(_γ\) 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 arrhythogenic Ca$^{2+}$ release.

**Methods**

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

**Mice and Surgical Procedure**

PI3K\(_γ\)-deficient mice (PI3K\(_γ^{−/−}\)) and knock-in mice with catalytically inactive PI3K\(_γ\) (PI3K\(_γ^{KD/KD}\)) 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\(_γ^{+/−}\)) were used as controls. Mechanical stress was imposed on the left ventricle by transverse aortic constriction between the truncus anonymous and the 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 fibroin-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-4-AM (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 PI3K\(_γ\) on catecholamine-induced arrhythmia, ECGs were recorded in PI3K\(_γ^{+/−}\), PI3K\(_γ^{−/−}\), and PI3K\(_γ^{KD/KD}\) 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\(_γ^{−/−}\) than in PI3K\(_γ^{+/−}\) and PI3K\(_γ^{KD/KD}\) animals (Figure 1A and Table I in the online-only Data Supplement). Interestingly, epinephrine-treated PI3K\(_γ^{−/−}\) mice displayed runs of premature ventricular beats, whereas no runs were observed in PI3K\(_γ^{+/−}\) and PI3K\(_γ^{KD/KD}\) animals (Figure 1B and 1C). These data indicate that the kinase-independent function of PI3K\(_γ\) regulates both the chronotropic and arrhythmogenic effects of myocardial β-AR stimulation.

PI3K\(_γ\) 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\(_γ^{−/−}\) animals (Figure
Compartmentalized PDE3 and PDE4

seconds) combined with the cAMP, Epac2-cAMPs22 (Figure 2A, insets). Activation of fluorescence resonance energy transfer sensor for intracellular evaluated in neonatal cardiomyocytes expressing the fluores-

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 PI3Kγ−/− than in PI3Kγ+/+ and PI3KγKD/KD cells (Figure 2B), demonstrating that cAMP hydrolysis by PDEs is impaired in the absence of PI3Kγ.

In adult cardiomyocytes, cAMP generated by β2-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 geno-
types (Figure IIIA–IIIC and Table II in the online-only Data Supplement), indicating that PDE3 is required to limit β2-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 PI3Kγ−/− than in PI3Kγ+/+ and PI3KγKD/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 β2-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 PI3Kγ−/− than in

Figure 1. Phosphoinositide 3-kinase γ (PI3Kγ) protects against β2-adrenergic receptor (AR)–induced ventricular arrhythmia. A, Heart rate of PI3Kγ+/+ (n = 7), PI3Kγ−/− (n = 7), and PI3KγKD/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 β2-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, Kaplan-Meier survival curve of PI3Kγ+/+ (n = 12), PI3Kγ−/− (n = 21), and PI3KγKD/KD (n = 8) mice 3 weeks after transverse aortic constriction (TAC). *P<0.01 by log-rank test. D, Representative ECG traces of a PI3Kγ−/− 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.

1B and 1C in the online-only Data Supplement). These data indicate that arrhythmias occurring in PI3Kγ−/− hearts are related to abnormal β2-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 PI3Kγ−/− (59% on day 7) than in PI3Kγ+/+ (8%) and PI3KγKD/KD (12%) mice (P<0.01; Figure 1D). Serial ECG monitoring of transverse aortic constriction–treated animals revealed that PI3Kγ−/− 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.

PI3Kγ Controls β2-AR/cAMP Responses Through Compartmentalized PDE3 and PDE4

The relation between PI3Kγ and β2-AR/cAMP signaling was evaluated in neonatal cardiomyocytes expressing the fluorescence resonance energy transfer sensor for intracellular cAMP, Epac2-cAMPS22 (Figure 2A, insets). Activation of β2-ARs by short application of isoproterenol (100 nmol/L, 15 seconds) combined with the β1-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 PI3Kγ−/− than in PI3Kγ+/+ and PI3KγKD/KD cells (Figure 2B), demonstrating that cAMP hydrolysis by PDEs is impaired in the absence of PI3Kγ.

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PI3Kγ^{+/+} and PI3Kγ^{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, PI3Kγ controls PDE3 and PDE4 in distinct subcellular compartments. Therefore, half-decay time was 3-fold higher in PI3Kγ^{KD/KD} cardiomyocytes, whereas PI3Kγ^{−/−} responses were abnormally prolonged (Figure IVA in the online-only Data Supplement). Accordingly, half-decay time was 3-fold higher in PI3Kγ^{−/−} than in PI3Kγ^{+/+} and PI3Kγ^{KD/KD} cardiomyocytes (Figure IVB in the online-only Data Supplement). Thus, PI3Kγ limits β2-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 PI3Kγ^{−/−} than in PI3Kγ^{+/+} and PI3Kγ^{KD/KD} cardiomyocytes (Figure VD and VE in the online-only Data Supplement), thus demonstrating that PDE4 fails to terminate subsarcolemmal β2-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 different subcellular compartments.

To further prove a major involvement of PI3Kγ scaffold function, β2-AR/cAMP responses were measured by a ICUE3 probe34 in cardiomyocytes expressing a kinase-dead mutant PI3Kγ (PI3Kγ^{KD-RFP}; Figure VIIA in the online-only Data Supplement). In PI3Kγ^{−/−} neonatal cardiomyocytes, transfected PI3Kγ^{KD-RFP} fully rescued β2-AR/cAMP responses to the levels of PI3Kγ^{+/+} cells (Figure VIIB and VIIIC in the online-only Data Supplement).

Together, these data unveil a critical role for the scaffold function of PI3Kγ in terminating β2-AR/cAMP signaling via compartmentalized modulation of PDE3 and PDE4.
PI3Kγ Activates PDE4A, PDE4B, and PDE3A via PKA

Different PDE3 and PDE4 isoenzymes are expressed in the myocardium. The specific isoforms regulated by PI3Kγ were thus analyzed in adult whole hearts. The catalytic activity of PDE4A and PDE4B was 20% lower in PI3Kγ−/− than in PI3Kγ+/+ and PI3Kγ<sup>KD/KD</sup> 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 3D and 3E). Two distinct PDE3A isoforms, 97 and 106 kDa also coprecipitated with PI3Kγ (Figure IXB in the online-only Data Supplement). These data indicate that PI3Kγ was not found to interact with PDE4D (Figure IXB in the online-only Data Supplement). In addition to PDE3B<sup>21</sup>, PDE3A activity was found to be 30% lower in PI3Kγ−/− than in PI3Kγ+/+ and PI3Kγ<sup>KD/KD</sup> 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 PI3Kγ−/− 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.<sup>19</sup> Because PKA also activates PDE3A<sup>35</sup> and long PDE4 isoforms,<sup>36</sup> 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 (PI3Kγ<sup>K126A,R130A</sup>) 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, PI3Kγ<sup>K126A,R130A</sup> 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 PI3Kγ<sup>K126A,R130A</sup> (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<sub>2</sub>-AR/cAMP responses via PKA-mediated activation of different PDEs.

cAMP-Mediated Phosphorylation of Ca<sub>1.2</sub> 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. PKA
mediated phosphorylation of the LTCC pore-forming subunit Cav1.2 was 3-fold higher in PI3K/H9253/H11002/H11002 cardiac myocytes after 2-AR activation (Figure 6A). Consistent with a major role of PI3K/H9253 in controlling sarcolemmal PDE4, Cav1.2 phosphorylation was significantly enhanced in PI3K/H9253/H11002/H11002 over PI3K/H9253/H11001/H11001 cardiac myocytes when the contribution of PDE4 was revealed by Cilostamide (Figure XI in the online-only Data Supplement). Moreover, PI3K was found to be physically associated with Cav1.2 (Figure 6B), further supporting the view that PI3K limits β2-AR/cAMP signaling at the sarcolemma in proximity of the LTCC.

At the SR, PKA phosphorylates RyR and phospholamban. Ser-2808 RyR phosphorylation was unchanged in PI3K/H9253/H11002/H11002 compared with PI3K/H9253/H11001/H11001 cardiac myocytes after 2-AR stimulation (Figure 6C). On the contrary, Ser-16 phospholamban phosphorylation was 2.3-fold higher in PI3K/H9253/H11002/H11002 than in PI3K/H9253/H11001/H11001 cardiac myocytes (Figure 6D). In addition, phospholamban phosphorylation was significantly higher in PI3K/H9253/H11002/H11002, further supporting the view that PI3K limits β2-AR/cAMP signaling at the sarcolemma in proximity of the LTCC.

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 PI3Kγ+/− 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 PI3Kγ+/− and PI3Kγ−/− 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.
PI3K<sup>γ</sup><sup>−/−</sup> than in PI3K<sup>γ</sup><sup>+/+</sup> 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 PI3K<sup>γ</sup>-activated PDE3 and PDE4 delimit 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<sup>γ</sup><sup>−/−</sup> cardiomyocytes on 2-AR activation and when PDE3 and PDE4 were selectively blocked (Figure XIIIA–XIIIC).

Together, these data indicate that PI3K<sup>γ</sup> affects key regulators of ventricular cardiomyocyte excitability by controlling local pools of β<sub>2</sub>-AR/cAMP.

**PI3K<sup>γ</sup>-Null Cardiomyocytes Develop Increased Spontaneous Ca<sup>2+</sup> Release Events**

CAMP-mediated phosphorylation of Ca<sub>1.2</sub> and phospholamban enhances LTCC current amplitude and accelerates SR Ca<sup>2+</sup>-reuptake, respectively. Previous evidence demonstrated that PI3K<sup>γ</sup><sup>−/−</sup> adult cardiomyocytes have higher LTCC current density than PI3K<sup>γ</sup><sup>+/+</sup> cells after β<sub>2</sub>-AR activation. To explore the role of PI3K<sup>γ</sup> in Ca<sup>2+</sup> homeostasis further, SR Ca<sup>2+</sup>-release was analyzed in quiescent and epinephrine-treated adult cardiomyocytes (Figure 7A). Ca<sup>2+</sup>-spark frequency was not significantly different between PI3K<sup>γ</sup><sup>+/+</sup> and PI3K<sup>γ</sup><sup>−/−</sup> cells after epinephrine (Figure 7B). In contrast, the effect of epinephrine on Ca<sup>2+</sup>-spark occurrence was higher in PI3K<sup>γ</sup><sup>−/−</sup> than in PI3K<sup>γ</sup><sup>+/+</sup> cardiomyocytes (Figure 7C), revealing a hyperresponsiveness of PI3K<sup>γ</sup><sup>−/−</sup> cells to adrenergic stimulation. In addition, Ca<sup>2+</sup>-spark amplitude was significantly increased in PI3K<sup>γ</sup><sup>−/−</sup> cardiomyocytes in basal conditions and further enhanced by adrenergic stimulation (Figure 7D). Thus, spontaneous SR Ca<sup>2+</sup>-release via RyR is enhanced in the absence of PI3K<sup>γ</sup>.

The impact of local Ca<sup>2+</sup>-mishandlings on global intracellular Ca<sup>2+</sup>-was evaluated next. Intracellular Ca<sup>2+</sup>-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 PI3K\(^\gamma\) prevents spontaneous Ca\(^{2+}\) release events after activation of β\(_2\)-ARs.

Together, these data demonstrate that PI3K\(^\gamma\) 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 PI3K\(^\gamma\) 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 PI3K\(^\gamma\) orchestrates multiprotein complexes controlling both PKA-mediated activation of PDEs (PDE3A, PDE4A, PDE4B) and a physiological feedback inhibition of the Ca\(_{\text{v}1.2}\) LTCC subunit and phospholamban.

The full rescue of ventricular arrhythmia with the β\(_2\)-AR antagonist ICI-118551 indicates a selective engagement of PI3K\(^\gamma\) 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 PI3K\(^\gamma\) also influences sinoatrial node function in vivo and supports previous evidence that PI3K\(^\gamma\) increases spontaneous pacemaker activity in isolated sinoatrial node myocytes.28
It has previously been reported that PI3K directly associates with PKA and acts as an A-kinase anchoring protein involved in the negative regulation of cardiac cAMP. The present study further demonstrates that PI3K 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 PI3K kinase activity and depends on protein scaffolding. Whether PI3K regulates PDE3 or PDE4 has been a subject of debate. In whole hearts, PI3K has been shown to regulate mainly PDE3B, independently of its kinase activity. In contrast, in isolated cardiomyocytes, PI3K appears to modulate PDE4 but not PDE3 activity. The present study provides a solution to this controversy in that PI3K 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 PI3K in the cytosol is PDE3. Conversely, PI3K controls PDE4- but not PDE3-dependent cAMP pools close to the plasma membrane.

The finding that PDE3 is not required for the modulation of β2-AR/cAMP signaling at the plasma membrane was unexpected because PDE3 isoenzymes are known to be membrane bound. However, PDE3 localizes mainly at the SR/endoplasmic reticulum rather than at the plasma membrane. In agreement with this idea, PDE3A was modulated by PI3K 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. Our findings demonstrate that the activity of these pools of PDE4A and PDE4B relies on PI3K scaffold activity. Taken together, present and previous data indicate that PI3K 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 PI3K operates a feedback loop inhibiting PKA-mediated phosphorylation of the Ca,1.2 subunit of LTCC on β-AR stimulation. This mechanism eventually explains the

Figure 8. Spontaneous Ca2+ release events are increased in phosphoinositide 3-kinase γ (PI3Kγ)-null (PI3Kγ−/−) cardiomyocytes. A, Representative traces of Ca2+ transients recorded in electrically paced (0.5 Hz) adult cardiomyocytes during stimulation with epinephrine (Epi; 100 nmol/L) or Epi plus the β2-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.
PI3K

Similarly, enhanced activation of LTCC is the main trigger of the Cav1.2 channel complex is another mechanism by which the finding that PI3K

Cav1.2 causing increased channel opening leads to severe proarrhythmic effect of uncontrolled LTCC function has also been shown in humans, in whom a missense mutation of Ca,1.2 suggests that PI3K

mediated control of PDE4B activity in the Ca,1.2 channel complex is another mechanism by which PI3K

confers protection against cardiac arrhythmia.

The effects of enhanced I,calc, detected in PI3Kγ-/- 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 PI3Kγ-/- cardiomyocytes. This is consistent with previous evidence that RyR activity is PI3Kγ independent but relies on the regulation of a complex containing PDE4D. 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 β2-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 β2-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 defect in PI3Kγ-directed protein complexes. Hence, deregulation of PI3Kγ scaffold function may constitute an important component of heart failure-related arrhythmias.

Acknowledgments

We wish to thank Valérie Domergue-Dupont and the animal core facility of IFR141 for efficient handling and preparation of the animals and Patrick Lechêne for skillful technical assistance.

Sources of Funding

This work was supported by grants from the Fondation Leducq 06CVD02 cycAMP (Drs Conti, Hirsch, and Fischmeister) and Fondation Leducq 09CD01 (Dr Hirsch), EU contract LSHM-CT-2005–018833/EUGeneHeart (Drs Hirsch and Fischmeister), Telethon (Dr Hirsch), Regione Piemonte (Dr Hirsch), CRT (Dr Hirsch), PRIN (Dr Hirsch), Agence Nationale pour la Recherche ANR-Geno-034 (Dr Gomez), CODDIM COD 100256 (Dr Gomez), and Agence Nationale pour la Recherche grant 2010 BLAN 1139 01 (Dr Vandecasteele). Dr Zahradniková is a fellow of Université Paris Sud.

Disclosures

None.

References


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


_Circulation_. 2012;126:2073-2083; originally published online September 24, 2012;
doi: 10.1161/CIRCULATIONAHA.112.114074
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Supplemental Material

*PI3K*γ Protects against Catecholamine-induced Ventricular Arrhythmia through PKA-mediated Regulation of Distinct Phosphodiesterases

*PI3K*γ protects against arrhythmia

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Supplemental Methods

All experiments were carried out according to the European Community guiding principles in the care and use of animals (86/609/CEE, CE Off J n°L358, 18 December 1986), the local ethics committee (CREEA Ile-de-France Sud) guidelines and the French decree n°87-848 of October 19, 1987 (J Off Rép Fr, 20 October 1987, pp. 12245–12248). Authorizations to perform animal experiments according to this decree were obtained from the Ministère Français de l’Agriculture, de la Pêche et de l’Alimentation (n°92-283, June 27, 2007).

Mice and surgical procedure

PI3Kγ-deficient mice (PI3Kγ−/−) and knock-in mice with catalytically inactive PI3Kγ (PI3KγKD/KD) were generated as previously described.1,2 Mutant mice were back-crossed with C57Bl/6 mice for 15 generations to inbreed the genetic background and C57Bl/6 mice were used as controls (PI3Kγ+/+). Mechanical stress was imposed to the left ventricle by transverse aortic constriction (TAC) between the truncus anonymous and the left carotid artery.2 The systolic pressure gradient and the degree of biomechanical stress were measured using a Vevo 2100 echocardiograph (VisualSonics, Toronto, Canada) at the level of the banding at the end of the treatment. Animals which displayed a pressure gradient lower than 60 mmHg or higher than 90 mmHg were excluded from further analyses.

ECG recording

For evaluation of epinephrine-induced arrhythmias, mice were anesthetized with 1% isoflurane and subjected to intra-peritoneal injection of the indicated drugs under continuous ECG monitoring using a Vevo 2100 echocardiograph (VisualSonics, Toronto, Canada).
TAC-treated animals, serial ECG monitoring was performed four times daily, for a total of four hours, starting on day three after surgery.

**Cardiomyocyte preparation**

Spontaneously beating neonatal cardiac myocytes were prepared from hearts of 1- to 2-day-old mouse pups. Briefly, hearts were quickly excised, atria were cut off and then ventricles were minced and pre-digested overnight at 4°C in calcium-free HEPES-buffered Hanks’ solution, pH 7.4, containing 0.5 mg/ml trypsin (USB Corporation, Cleveland, Ohio). The following day, ventricles were digested at 37°C for 5 minutes in calcium-free HEPES-buffered Hanks’ solution, pH 7.4, containing 330 U/ml collagenase type II (Worthington Biochemical, Lakewood, NJ). The digestion step was repeated 3-4 times. To reduce the contribution of non-myocardial cells, cells were pre-plated for 1h. The myocyte-enriched cells remaining in suspension were plated in 35-mm tissue culture dishes at a density of $1.5 \times 10^5$ cells per dish. Culture dishes were pre-coated with a solution of 0.2% gelatin (Sigma-Aldrich, Saint Louis, MO) and 15 mg/ml fibronectin for 30 minutes. Myocytes were cultured in a Dulbecco’s modified Eagle’s medium/Medium 199 (Gibco, Carlsbad, CA) mix containing 10% horse serum, 5% fetal bovine serum, and 5 mmol/L penicillin/streptomycin (Gibco, Carlsbad, CA).

Ventricular cardiomyocytes were isolated from 4 month-old mice as previously described.³

**Cell infection and transfection**

Twenty-four hours after isolation, mouse neonatal cardiomyocytes were infected with an adenovirus encoding Epac2-camps⁴ or pm-Epac2-camps⁵ (MOI = 1000 pfu/cell). Live cell imaging was performed 24 hours following adenovirus infection.
Co-transfection of neonatal cardiomyocytes with ICUE3\textsuperscript{6} and either PI3KγKD-RFP or RFP was carried out by electroporation using a Nucleofector device (AMAXA, Gaithersburg, MD), according to the manufacturer’s protocol. Briefly, freshly isolated cells (2 x 10\textsuperscript{6}) were suspended in 100 µL of nucleofection solution (VPE-1002, AMAXA, Gaithersburg, MD), mixed with 1 µg of ICUE3-pcDNA3 and 1 µg of either pmRFP-C1-PI3KγKD-RFP or pmRFP-C1, and subjected to electroporation in a Nucleofector (program G-009). Cells were then plated on fibronectin/gelatin-coated FRET dishes and live cell imaging was performed 24 hours after transfection.

HEK293 cells were transfected with the indicated plasmids (up to 10 µg of total cDNA) with calcium phosphate. 24 hours after transfection cells were lysed as described below.

**FRET imaging and analysis**

Cardiomyocytes were maintained in a K\textsuperscript{+}-Ringer solution containing (in mmol/L) 121.6 NaCl, 5.4 KCl, 1.8 MgCl\textsubscript{2}, 1.8 CaCl\textsubscript{2}, 4 NaHCO\textsubscript{3}, 0.8 NaH\textsubscript{2}PO\textsubscript{4}, 5 D-glucose, 5 sodium pyruvate, 10 HEPES, adjusted to pH 7.4.

For imaging of cell expressing Epac2-camps\textsuperscript{4} or pm-Epac2-camps\textsuperscript{5}, images were captured every 5 s using the 40x oil immersion objective of a Nikon TE 300 inverted microscope connected to a software-controlled (Metaflour, Molecular Devices, Sunnyvale, CA) cooled charge coupled (CCD) camera (Sensicam PE; PCO, Kelheim, Germany). CFP was excited during 150-300 ms by a Xenon lamp (100W, Nikon, Champigny-sur-Marne, France) using a 440/20BP filter and a 455LP dichroic mirror. Dual emission imaging of CFP and YFP was performed using an Optosplit II emission splitter (Cairn Research, Faversham, UK) equipped with a 495LP dichroic mirror and BP filters 470/30 and 535/30, respectively. Average fluorescence intensity was measured in a region of interest comprising the entire cell.
or a significant part of the cell. Background was subtracted and YFP intensity was corrected for CFP spillover into the 535 nm channel before calculating the CFP/YFP ratio. Ratio images were obtained by using Image J software (National Institute of Health). \( \tau \) decay values were determined by fitting the decrease phase of cAMP transients with the following monoexponential equation: 
\[
y(t) = A \cdot \exp(-t/\tau_{\text{decay}}) + B.
\]
Origin 6 software was used for \( \tau \) decay calculation (Microcal software, Northampton, MA). T-50% decay values were determined using Microsoft Excel software.

CAMP imaging of cardiomyocytes expressing either PI3K\(\gamma\)KD-RFP or RFP was allowed by co-expression of ICUE3\(^6\), a FRET sensor for cAMP that carries cpVenus instead of pEYFP and thus shows a larger dynamic range than Epac2-camps.\(^7\) Imaging was performed using a Leica TCS SP5 system (Leica Microsystems Inc, Buffalo Grove, IL, USA) with an argon laser using a 63x oil-immersion lens. Excitation wavelengths were 458 and 514 nm for CFP and YFP, respectively. Images were taken every 4 s without any line averaging. Images were captured at a scanning speed of 400 MHz and a pixel resolution of 512 x 512. FRET efficiency was calculated by the Leica application wizard for FRET-sensitized emission imaging, according to method 3: 
\[
E_A(i) = \frac{B}{A},
\]
where \( E_A(i) \) is the apparent FRET efficiency; \( A \) and \( B \) are the intensities of the CFP and FRET channels, respectively.

Ca\(^{2+}\) measurements

For Ca\(^{2+}\) spark measurements, adult cardiomyocytes were loaded with the Ca\(^{2+}\) fluorescence dye fluo-4AM (Molecular Probes, Invitrogen Corporation, Carlsbad, CA, USA) as previously reported\(^8\) and Ca\(^{2+}\) sparks were visualized in quiescent myocytes by a Leica SP5 confocal microscope (Leica Microsystems Inc, Buffalo Grove, IL, USA) using the 488 nm line of an argon laser for excitation and collecting emission at >505 nm. During recording, cells were bathed in a Tyrode solution containing (in mmol/L) 140 NaCl, 4 KCl, 1.1 MgCl\(_2\),
10 HEPES, 10 glucose, 1.8 CaCl₂ adjusted to pH=7.4. Images were analyzed by IDL (RSI Inc.) using homemade routines.⁸

For Ca²⁺ transient measurements, cardiomyocytes were loaded with 5 µM Fura-2 AM (Molecular Probes, Invitrogen Corporation, Carlsbad, CA, USA) at room temperature for 15 minutes and then washed with the K⁺-Ringer solution as described above for FRET imaging. Loaded cells were field-stimulated (5 V, 4 ms) at a frequency of 0.5 Hz. Fura-2 ratio (measured at 512 nm upon excitation at 340 nm and 380 nm) were simultaneously recorded using an IonOptix System (IonOptix, Milton, MA, USA). Ca²⁺ transients were assessed by the percentage of variation of the Fura-2 ratio by dividing the twitch amplitude (difference of end-diastolic and peak systolic ratios) to end-diastolic ratio. Arrhythmia occurrence was assessed by calculating the number of spontaneous calcium release (SCR) events during 20s. All parameters were calculated offline using a dedicated software (IonWizard 6x, IonOptix).

Protein extraction and immunoprecipitation

Total membranes and cytosolic fractions were prepared by homogenization of liquid nitrogen-frozen hearts in 120 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), protease inhibitor Complete (Roche Applied Science, Indianapolis, IN) and phosphatase inhibitors (50 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, and 10 mmol/L sodium pyrophosphate). Lysates were incubated on ice for 30 min and then centrifuged at 3000 rpm for 5 min at 4°C. Membrane and cytosolic fractions were then separated by centrifugation of supernatants at 38000 rpm for 1 h at 4°C in a SW55Ti rotor (Beckman Coulter). Supernatants (cytosolic fraction) were collected and pellets (membrane fraction) were solubilized in ice-cold lysis buffer, supplemented with 1% Triton X-100. Protein concentration was determined by Bradford method and extracts were either used for Western blotting or first subjected to immunoprecipitation and then assayed for PDE activity.
For total heart lysates preparation, hearts were homogenized in 120 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0) and 1% Triton X-100, supplemented with protease and phosphatase inhibitors, as described above. After 30 min incubation on ice, lysates were centrifuged at 3000 rpm for 5 min at 4°C and either used for Western blotting or subjected to immunoprecipitation/PDE activity measurements.

For protein extraction from cardiomyocytes, 48 hours after plating cells were stimulated with the indicated drugs and immediately scraped in ice-cold buffer containing 50 mmol/L HEPES pH 7.5, 400 mmol/L NaCl, 1.5 mmol/L EGTA, 10% glycerol, 1% Triton X-100, supplemented with protease and phosphatase inhibitors. After 15 min incubation on ice, lysates were centrifuged at 10000g for 10 min at 4°C and supernatants were used directly for Western blotting.

Transfected HEK293 cells were scraped in 120 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0) and 1% Triton X-100, supplemented with protease and phosphatase inhibitors, as described above. Detergent-insoluble material was precipitated by centrifugation at 13000 rpm for 15 min at 4°C. Supernatants were subjected to immunoprecipitation and then assayed for PDE activity or Western blotting.

For immunoprecipitation assays, pre-cleared extracts were incubated with 20 µl of a 1:1 slurry of protein A- or G-Sepharose (Amersham Biosciences, Buckinghamshire, UK) and 1 µg of antibody/mg of protein for 2 h at 4°C. Immunocomplexes were then extensively washed with lysis buffer and either used for Western blotting or subjected to PDE activity assay.

**PDE assay**

PDE activity in immunoprecipitates was measured according to the two-step method of Thompson and Appleman\(^9\) as previously described\(^10\), with minor modifications. In brief,
immunoprecipitations were assayed in a total volume of 200 µl of reaction mixture containing 40 mmol/L Tris-HCl (pH 8.0), 1 mmol/L MgCl₂, 1.4 mmol/L 2-mercapto-ethanol, 1 µmol/L cAMP (Sigma-Aldrich, Saint Louis, MO) and 0.1 µCi of [³H]cAMP (Amersham Bioscience, Buckinghamshire, UK) for 30 min at 33°C. To stop the reaction samples were boiled at 95°C for 3 min. The PDE reaction product 5'-AMP was then hydrolyzed by incubation of the assay mixture with 50 µg of Crotalus Atrox snake venom for 15 min at 37°C (Sigma-Aldrich, Saint Louis, MO). The resulting adenosine was separated by anion exchange chromatography using 400 µl of a 30% (w/v) suspension of Dowex AG1-X8 resin (Bio-Rad, Segrate, Milano, Italy). The amount of radiolabelled adenosine in the supernatant was quantitated by scintillation counting (Ultima Gold scintillation liquid from Perkin Elmer, Waltham, MA).

**Antibodies, plasmids and reagents**

Monoclonal antibodies against p110γ were used as previously described¹. A new mouse monoclonal antibody against p110γ was produced and used for immunoprecipitation as previously reported.¹¹ PDE4A, PDE4B and PDE4D were immunoprecipitated and detected by Western blotting using rabbit polyclonal antibodies as previously described.¹⁰ The mouse monoclonal antibody against PDE3A was used as previously described.¹² Anti-Phospho-Troponin I (cardiac) Ser-23/24 and Troponin I antibodies were purchased from Cell Signaling Technology (Danvers, MA); anti-Phospho-Phospholamban Ser-16 and Phospholamban antibodies were from Millipore (Billerica, MA). Anti-phospho-RyR2 Ser-2808 rabbit polyclonal antibody was a kind gift from Andrew Marks (Columbia University, New York, NY, USA). The anti-RyR monoclonal antibody was from Pierce Antibodies (Pierce, Rockford, IL, USA). Anti-Ca₉,1.2 and Phospho-Ca₉,1.2 rabbit polyclonal antibodies were used as described.¹³ Antibodies against the regulatory and catalytic subunits of PKA were from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA).
ICUE3-pcDNA3 was described previously.\textsuperscript{6} PI3K\textgamma WT-pcDNA3 was a generous gift from Dr. Reinhard Wetzker (Friedrich Schiller University, Jena, Germany). PI3K\textgamma K126A,R130A-pcDNA3 was generated previously.\textsuperscript{11} The PDE3A expression vector was a generous gift from Dr. Miles Houslay (University of Glasgow, Glasgow, UK). For over-expression of PDE4B3, the ORF of human PDE4B3 was cloned into the pTARGET vector. PI3K\textgamma KD was subcloned from pcDNA3 into a pmRFP-C1 vector.

Isoproterenol, CGP-20712A, ICI-118551 and epinephrine were all from Sigma (Sigma-Aldrich, Saint Louis, MO). Myristoylated-PKI and Ro-201724 were purchased from Merck Millipore (Merck KGaA, Darmstadt, Germany). Cilostamide was from Tocris Bioscience (Tocris Bioscience, Ellisville, MI).

**Statistical analysis**

Prism software (GraphPad software Inc., La Jolla, CA, USA) was used for statistical analysis. \( P \) values were calculated using Kruskall-Wallis non parametric test followed by Dunn’s post hoc analysis and non parametric Mann-Whitney test when appropriate. Fisher exact test was used to evaluate arrhythmia incidence and Logrank test was used for survival analysis.
### Supplemental Tables

**Supplemental Table 1.** Chronotropic responses of PI3K$^{+/+}$ and PI3K$^{-/-}$ mice to epinephrine and ICI-118551.

<table>
<thead>
<tr>
<th>Heart rate (bpm)</th>
<th>Basal</th>
<th>Epi</th>
<th>ICI</th>
<th>Epi + ICI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI3K$^{+/+}$</td>
<td>397.8 ± 12.5</td>
<td>560.9 ± 3.5**</td>
<td>399.3 ± 29.3</td>
<td>570.5 ± 1.8†††</td>
</tr>
<tr>
<td>PI3K$^{-/-}$</td>
<td>393.7 ± 13.6</td>
<td>620.7 ± 14.6***‡</td>
<td>390.0 ± 3.5</td>
<td>533.8 ± 16.0††§</td>
</tr>
</tbody>
</table>

Mice were treated with epinephrine (Epi, 2 mg/Kg i.p.) and/or ICI-118551 (ICI, 2 mg/Kg i.p.). All data (heart rate, indicated as beats per minute) are expressed as mean ± SEM.  
**$P<0.01$ and ***$P<0.001$ versus basal. ††$P<0.01$ and †††$P<0.001$ versus ICI. ‡$P<0.01$ versus PI3K$^{+/+}$(Epi). §$P<0.001$ versus Epi. Basal/Epi: PI3K$^{+/+}$ n=7 and PI3K$^{-/-}$ n=6; ICI/Epi + ICI: PI3K$^{+/+}$ n=6 and PI3K$^{-/-}$ n=6.
Supplemental Table 2. Decay kinetics of β2-AR/cAMP transients measured with Epac2-camps.

<table>
<thead>
<tr>
<th>τ decay (s)</th>
<th>Iso+CGP</th>
<th>Iso+CGP+Cil</th>
<th>Iso+CGP+Ro</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI3Kγ+/+</td>
<td>47.9 ± 2.7</td>
<td>105.4 ± 8.6***</td>
<td>135.6 ± 14.0***</td>
</tr>
<tr>
<td>PI3KγKD/KD</td>
<td>48.8 ± 2.4</td>
<td>106.7 ± 6.1***</td>
<td>124.1 ± 13.8***</td>
</tr>
<tr>
<td>PI3Kγ−/−</td>
<td>61.8 ± 4.5†</td>
<td>147.7 ± 15.6***†</td>
<td>259.0 ± 34.4***†</td>
</tr>
</tbody>
</table>

τ decay (s) was determined by fitting cAMP transient decay with a mono-exponential function. All data are expressed as mean ± SEM. ***P<0.001 versus Iso + CGP. †P<0.05 versus either PI3Kγ+/+ or PI3KγKD/KD within the same group. Iso = isoproterenol (100 nmol/L); CGP = CGP-20712A (100 nmol/L); Cil = cilostamide (1 µmol/L); Ro = Ro-201724 (10 µmol/L).
Supplemental Table 3. Decay kinetics of β2-AR/cAMP transients measured with a plasma membrane-targeted FRET sensor for cAMP (pm-Epac2).

<table>
<thead>
<tr>
<th></th>
<th>T-50% decay (s)</th>
<th>Iso+CGP</th>
<th>Iso+CGP+Cil</th>
<th>Iso+CGP+Ro</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI3Kγ+/+</td>
<td>45.6 ± 4.1</td>
<td>64.2 ± 12.7</td>
<td>315.5 ± 50.9***</td>
<td></td>
</tr>
<tr>
<td>PI3KγKD/KD</td>
<td>49.7 ± 3.7</td>
<td>64.7 ± 11.8</td>
<td>379.7 ± 89.2***</td>
<td></td>
</tr>
<tr>
<td>PI3Kγ−/−</td>
<td>122.7 ± 29.8††</td>
<td>276.9 ± 103.4†††</td>
<td>344.1 ± 203.0</td>
<td></td>
</tr>
</tbody>
</table>

T-50% decay (s) for cAMP transient was measured as time from the peak to 50% decay. All data are expressed as mean ± SEM. ***P<0.001 versus Iso + CGP. ††P<0.01 and †††P<0.001 versus either PI3Kγ+/+ or PI3KγKD/KD within the same group. Iso = isoproterenol (100 nmol/L); CGP = CGP-20712A (100 nmol/L); Cil = cilostamide (1 µmol/L); Ro = Ro-201724 (10 µmol/L).
Supplemental Figure Legends

Supplemental Figure 1. PDE3 and PDE4 are necessary and sufficient to terminate β₂-AR/cAMP responses. A-C, FRET traces (squares) obtained from PI3Kγ⁺/⁺ (A, n=11) PI3Kγ⁻/⁻ (B, n=9) and PI3KγKD/KD (C, n=16) cardiomyocytes treated with: isoproterenol (Iso, 100 nmol/L, 15 seconds), the β₁-AR antagonist CGP-20712A (CGP, 100 nmol/L), the PDE3 inhibitor Cilostamide (Cil, 1 µmol/L) and the PDE4 inhibitor Ro-201724 (Ro, 10µmol/L). Circles represent the FRET traces obtained with Iso + CGP alone. D, Superimposition of average traces reported in A, B and C. Error bars indicate SEM.

Supplemental Figure 2. Effect of PDE3 inhibition on β₂-AR/cAMP responses. A-C, FRET traces (squares) obtained from PI3Kγ⁺/⁺ (A, n=21), PI3Kγ⁻/⁻ (B, n=16) and PI3KγKD/KD (C, n=24) cardiomyocytes treated with isoproterenol (Iso, 100 nmol/L, 15 seconds), the β₁-AR antagonist CGP-20712A (CGP, 100 nmol/L) and the PDE3 inhibitor Cilostamide (Cil, 1 µmol/L). Circles show the effect of Iso + CGP alone. Error bars indicate SEM.

Supplemental Figure 3. Effect of PDE4 inhibition on β₂-AR/cAMP responses. A-C, FRET traces (squares) obtained from PI3Kγ⁺/⁺ (A, n=12), PI3Kγ⁻/⁻ (B, n=27) and PI3KγKD/KD (C, n=15) cardiomyocytes treated with isoproterenol (Iso, 100 nmol/L, 15 seconds), the β₁-AR antagonist CGP-20712A (CGP, 100 nmol/L) and the PDE4 inhibitor Ro-201724 (Ro, 10 µmol/L). Circles show the effect of Iso + CGP alone. Error bars indicate SEM.

Supplemental Figure 4. PI3Kγ limits subsarcolemmal β₂-AR/cAMP responses. A, Near membrane FRET traces obtained from PI3Kγ⁺/⁺ (A, n=15), PI3Kγ⁻/⁻ (B, n=6) and PI3KγKD/KD (C, n=16) cardiomyocytes treated with isoproterenol (Iso, 100 nmol/L, 15 seconds) plus the
β₁-AR antagonist CGP-20712A (CGP, 100 nmol/L). The plasma membrane-targeted cAMP FRET sensor was pm-Epac2-camps. Insets show representative CFP and YFP images. B, Decay kinetics (T-50% decay) of cAMP responses shown in A. In A, error bars indicate SEM. **P<0.01 and ***P<0.001.

Supplemental Figure 5. Effect of PDE3 inhibition on subsarcolemmal β₂-AR/cAMP responses. A-C, FRET traces (squares) obtained from PI3Kγ⁺/⁺ (A, n=10), PI3Kγ⁻/⁻ (B, n=5) and PI3Kγₘₙ/Kₘₙ (C, n=14) cardiomyocytes treated with isoproterenol (Iso, 100 nmol/L, 15 seconds), the β₁-AR antagonist CGP-20712A (CGP, 100 nmol/L) and the PDE3 inhibitor Cilostamide (Cil, 1 µmol/L). The plasma membrane-targeted cAMP FRET sensor was pm-Epac2-camps. Circles show the effect of Iso + CGP alone. D, Superimposition of average traces reported in A, B and C. E, Decay kinetics (T-50% decay) of cAMP responses shown in D. In A-D, error bars indicate SEM. ***P<0.001.

Supplemental Figure 6. Effect of PDE4 inhibition on subsarcolemmal β₂-AR/cAMP responses. A-C, FRET traces (squares) obtained from PI3Kγ⁺/⁺ (A, n=8), PI3Kγ⁻/⁻ (B, n=5) and PI3Kγₘₙ/Kₘₙ (C, n=8) cells treated with isoproterenol (Iso, 100 nmol/L, 15 seconds), the β₁-AR antagonist CGP-20712A (CGP, 100 nmol/L) and the PDE4 inhibitor Ro-201724 (Ro, 10µmol/L). The plasma membrane-targeted cAMP FRET sensor was pm-Epac2-camps. Circles show the effect of Iso + CGP alone. D, Superimposition of average traces reported in A, B and C. E, Decay kinetics (T-50% decay) of cAMP responses shown in D. In A-D, error bars indicate SEM.
**Supplemental Figure 7.** PI3KγKD-RFP rescues the defective cAMP clearance of PI3Kγ−/− cardiomyocytes. A, Representative CFP, YFP and RFP images of neonatal cardiomyocytes transfected with the FRET probe for cAMP ICUE3 and PI3KγKD-RFP. B, FRET traces obtained from PI3Kγ+/+ (circles) and PI3Kγ−/− (squares) cells expressing either PI3KγKD-RFP or RFP and treated with isoproterenol (Iso, 100 nmol/L) and the β1-AR antagonist CGP-20712A (CGP, 100 nmol/L). C, Maximal FRET changes (%) of cAMP responses shown in B. In B, error bars indicate SEM. *P<0.05. PI3Kγ+/+ + RFP n=5; PI3Kγ+/+ + PI3KγKD-RFP n=5; PI3Kγ−/− + RFP n=4; PI3Kγ−/− + PI3KγKD-RFP n=4.

**Supplemental Figure 8.** cAMP PDE activity of cytosolic and total PDE4A, PDE4B and PDE3A. A-C, cAMP PDE activity precipitated with selective anti-PDE4A (A), PDE4B (B) and PDE3A (C) antibodies from total lysates (left panel) and from cytosolic fractions (right panel) of PI3Kγ+/+, PI3Kγ−/− and PI3KγKD/KD hearts. n≥4 independent experiments.

**Supplemental Figure 9.** PI3Kγ neither binds nor activates PDE4D. A, cAMP PDE activity precipitated with a selective anti-PDE4D antibody from total heart lysates, total membranes and cytosolic fractions of PI3Kγ+/+, PI3Kγ−/− and PI3KγKD/KD hearts. n≥4 independent experiments. B, Western blot detection of PI3Kγ in PDE4D immunoprecipitates from PI3Kγ+/+ and PI3Kγ−/− hearts. A representative blot of three independent experiments is shown.

**Supplemental Figure 10.** PI3Kγ does not affect the subcellular distribution of PDE4A, PDE4B and PDE3A. Western blot detection of PDE4A (A), PDE4B (B) and PDE3A (C) in total membrane (m) and cytosolic (c) fractions from PI3Kγ+/+, PI3Kγ−/− and PI3KγKD/KD hearts. Bip and Cyclin E were used as markers for membranes and cytosol, respectively. Representative blots are provided.
**Supplemental Figure 11.** Effect of PDE3 inhibition on Ca\textsubscript{v}1.2 phosphorylation. PKA-mediated phosphorylation of Ca\textsubscript{v}1.2 in PI3K\textsubscript{γ}\textsuperscript{+/+} and PI3K\textsubscript{γ}\textsuperscript{−/−} cardiomyocytes treated with isoproterenol (Iso, 100 nmol/L), the β\textsubscript{1}-AR antagonist CGP-20712A (CGP, 100 nmol/L) and the PDE3 inhibitor Cilostamide (Cil, 1 µmol/L). A representative blot is shown. n≥3 independent experiments. **P<0.01.

**Supplemental Figure 12.** Effect of PDE3 and PDE4 inhibition on PLB phosphorylation. PKA-mediated phosphorylation of PLB in PI3K\textsubscript{γ}\textsuperscript{+/+} and PI3K\textsubscript{γ}\textsuperscript{−/−} cardiomyocytes treated with isoproterenol (Iso, 100 nmol/L), the β\textsubscript{1}-AR antagonist CGP-20712A (CGP, 100 nmol/L) and the PDE3 inhibitor Cilostamide (Cil, 1 µmol/L) (A) or the PDE4 inhibitor Ro-201724 (Ro, 10 µmol/L) (B). Representative blots are provided. n≥3 independent experiments. *P<0.05 and **P<0.01.

**Supplemental Figure 13.** TnI is hyper-phosphorylated upon β\textsubscript{2}-AR activation in PI3K\textsubscript{γ}\textsuperscript{−/−} cardiomyocytes. PKA-mediated phosphorylation of TnI in PI3K\textsubscript{γ}\textsuperscript{+/+} and PI3K\textsubscript{γ}\textsuperscript{−/−} cardiomyocytes treated with isoproterenol (Iso, 100 nmol/L) and the β\textsubscript{1}-AR antagonist CGP-20712A (CGP, 100 nmol/L) alone (A) or combined with the PDE3 inhibitor Cilostamide (Cil, 1 µmol/L) (B) or the PDE4 inhibitor Ro-201724 (Ro, 10 µmol/L) (C). Representative blots are provided. n≥4 independent experiments. *P<0.05.
**Supplemental References**


Supplemental Figure 1

A  

B  

C  

D
Supplemental Figure 2

A. PI3Kγ⁺/⁺

B. PI3Kγ⁻/⁻

C. PI3KγKD/KD
Supplemental Figure 4

A

Time (s)

0 50 100 150 200 250 300

0 2 4 6 8

Increase in CFP/YFP (%)

Iso

CGP

B

**

***

T-50% decay (s)

0 50 100 150 200 250

PI3K^+/+

PI3K^-/

PI3K^[K/DKD]
Supplemental Figure 7

A

![Images of YFP, CFP, and RFP]

B

Time (s)

Apparent FRET Efficiency (%)

Iso + CGP

C

Apparent FRET Efficiency (%)

RFP

PI3KγKD

- – + – +

PI3KγKD-RFP

+ – + – +

PI3Kγγγ

PI3Kγγγ
Supplemental Figure 8

A  
IP: PDE4A

B  
IP: PDE4B

C  
IP: PDE3A
Supplemental Figure 9

A

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PDE activity (cpm*10^6/IP)

B

IP: PDE4D

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116 — PI3Kγ

96 — PDE4D

WB
Supplemental Figure 11

P-Cav1.2/tot Cav1.2

P-Ca,1.2
(Ser-1928)

Tot Ca,1.2

WB

Iso + +
CGP + +
Cil + +

PI3Kγ+/+
PI3Kγ−/−

CGP

Cil

250

250

0

1

2

3

4

5

P-Cav1.2/tot Cav1.2 (fold over control)

**

**
Supplemental Figure 12

A

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P-PLB (Ser-16)

B

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P-PLB (Ser-16)
Supplemental Figure 13

A

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P-TnI (Ser-23/24)

Tot TnI

WB

B

C

Legend

* p < 0.05 compared to control

Note: Iso = isoproterenol, CGP = CGP12177, Cil = cilostamide, Ro = rottlerin.