EPAC AND PHOSPHOLIPASE Cε REGULATE CA^{2+} RELEASE IN THE HEART BY
ACTIVATION OF PROTEIN KINASE Cε AND CALCIUM-CALMODULIN-KINASEII
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Recently, we identified a novel signaling pathway involving Epac, Rap, and PLCε that plays a critical role in maximal β β β β-adrenergic receptor (BAR) stimulation of Ca^{2+}-induced-Ca^{2+} release (CICR) in cardiac myocytes. Here we demonstrate that PLCε phosphatidylinositol-4,5-bisphosphate (PIP2) hydrolytic activity and PLCε-stimulated Rap1 GEF activity are both required for PLCε-mediated enhancement of sarcoplasmic reticulum (SR) Ca^{2+} release and that PLCε significantly enhances Rap activation in response to βBAR stimulation in the heart. Downstream of PLCε hydrolytic activity, pharmacological inhibition of PKC significantly inhibited both βBAR- and Epac-stimulated increases in CICR in PLCε+/+ myocytes but had no effect in PLCε−/− myocytes. βBAR and Epac activation caused membrane translocation of PKCε in PLCε+/+ but not PLCε−/− myocytes and siRNA-mediated PKCε knockout significantly inhibited both βBAR and Epac mediated CICR enhancement. Further downstream, the Ca^{2+}/calmodulin-dependent protein kinase II (CamKII) inhibitor, KN93, inhibited βBAR- and Epac-mediated CICR in PLCε+/+ but not PLCε−/− myocytes. Epac activation increased CamKII Thr286 phosphorylation and enhanced phosphorylation at CamKII phosphorylation sites on the ryanodine receptor (Ryr2) (Ser2815) and phospholamban (PLB) (Thr17) in a PKC-dependent manner. Perforated patch clamp experiments revealed that basal and βBAR stimulated peak L-type current density are similar in PLCε+/+ and PLCε−/− myocytes suggesting that control of SR Ca^{2+} release, rather than Ca^{2+} influx through L-type Ca^{2+} channels, is the target of regulation of a novel signal transduction pathway involving sequential activation of Epac, PLCε, PKCε and CamKII downstream of βBAR-activation. These results provide a detailed characterization of the signaling mechanism underlying PLCε-dependent regulation of cardiac CICR downstream of the β-adrenergic receptor.

Phospholipase C (PLC) mediated hydrolysis of phosphatidylinositol (4,5) bisphosphate (PIP2) results in inositol-triphosphate (IP3) mediated Ca^{2+} release from intracellular stores and diacylglycerol-mediated activation of protein kinase C. This ubiquitous signaling pathway plays an integral role in regulating many physiological processes, including those of the cardiovascular system. PLCε is a recently-identified bi-functional PLC isoform that possesses both PIP2 hydrolytic and Rap guanine nucleotide exchange factor (GEF)
activity (1-4). The activity of PLCε is uniquely regulated by direct binding of small G-proteins including Ras, Rap, and Rho (5;6). PLCε activity is also stimulated by the heterotrimeric G-protein subunits Gαs, Gβγ and Gα12/13 (5;7;8) but direct binding of these subunits to PLCε has not been demonstrated. In primary astrocytes isolated from PLCε+/+ and PLCε−/− mice, multiple G protein-dependent upstream signals rely critically on PLCε-dependent generation of IP3 and DAG (9).

We recently discovered a surprising role for PLCε regulation downstream of the β-adrenergic receptor (βAR) in cardiac myocytes (10). Compared to normal mice, PLCε−/− mice exhibit reduced left ventricular developed pressure in response to strong βAR stimulation (10). This deficit results from a decrease in isoproterenol (Iso)-dependent stimulation of electrically-evoked Ca2+ release from the sarcoplasmic reticulum (SR) in single ventricular cardiac myocytes. βAR-stimulation increases cardiac Ca2+ release in a cAMP/protein kinase A (PKA)-dependent mechanism through phosphorylation of multiple targets of the cardiac excitability and Ca2+ handling machinery (11). Recently, we identified a PKA-independent, PLCε-mediated pathway that contributes to maximal Iso-dependent enhancement of Ca2+-induced-Ca2+ release (CICR) in cardiac myocytes (12) and explains the decreased βAR function in PLCε−/− mice. This novel pathway requires cAMP-dependent activation of the RapGEF, Epac (13), which subsequently stimulates Rap-dependent activation of PLCε.

We previously demonstrated that a novel Epac/PLCε pathway contributes to increased CICR during βAR signaling in the heart. Here, we establish a mechanistic link between PLCε activity and CICR by showing that Epac/Rap/PLCε-mediated enhancement of CICR in the heart requires both PLCε-PIP2 hydrolytic and PLCε-RapGEF activities and that downstream of PLCε, both PKCε and CamKII are required for Epac-dependent enhancement of Ca2+ release. In addition, voltage clamp experiments reveal that Iso-dependent activation of the Epac/PLCε pathway in the heart does not significantly alter Ca2+ influx through L-type Ca channels indicating that Ca2+ release from the sarcoplasmic reticulum is the ultimate target of this pathway.

**Experimental Procedures**

**Isolation of Cardiac Myocytes** - Adult ventricular cardiomyocytes (AVM) were isolated from male, 4 to 6 month-old wild type or PLCε−/− mice (C57/B6 background) as previously described (10). Briefly, mice were anaesthetized with ketamine (100 mg/kg body weight) and xylazine (5 mg/kg body weight) by IP injection. Hearts were excised and digested by Langendorff perfusion using either Liberase Blendzyme I (Roche) or a mixture of Collagenase A and D (Roche). Cells were plated on laminin (BD Biosciences)-coated coverslips or 35mm tissue culture dishes in minimum essential medium (MEM) supplemented with 2 mM L-glutamine, 2.5% fetal bovine serum (Hyclone), 1% penicillin/streptomycin, and 2.5 µM blebbistatin (Sigma) to prevent contractile activity.

**Transduction of AVM with Adenovirus** - Adenoviruses were prepared using the AdEasy system (Stratagene) with the mCMV promoter used to drive expression of YFP, PLCε WT, PLCεH1460L, PLCεΔCDC25, or PLCεK2150E. For wild type and domain mutant PLCε adenoviruses, a second mCMV promoter was used to drive the expression of YFP. Adult AVM were isolated and adhered to laminin-coated coverslips for 2 hours pre-infection. Plating media was removed and replaced with fresh media containing 300
m.o.i. of either YFP control, wild type PLCε, or PLCε domain mutant adenovirus. After 2 hours, the virus was removed and fresh media was added to the cells. The appearance of YFP fluorescence was used to determine the percentage of cells transduced at 24 hours post-infection. PLC message was measured by semiquantitative PCR and protein was detected by western blotting.

**PCR** For detection of PLCε mRNA in PLCε+/− AVMs transduced with either wild type or domain mutant PLCε adenovirus constructs, total RNA was isolated using the RNAeasy mini kit (Qiagen, Inc., Valencia, CA) following manufacturer recommendations. The Superscript III RT-PCR kit (Invitrogen) was used with 100 ng of total RNA template for RT-PCR reactions with mouse PLCε primers 5'-ACCCTGCAGGATAATGGTCTG-3' and 5'-ATGTGAATTCCGTGCTACCC-3' to yield a 300-bp product. GAPDH primers 5'-CAACGGGAAGCCCATCACCAT-3' and 5'-CCTTGGCAGCACCAGTGATGC-3' yielding a 350-bp product was used as control. RT was performed for 30 min at 42 °C followed by incubation at 94 °C for 2 min. The PCR parameters were denaturation at 94 °C for 30 s, annealing at 45 °C for 45 s, and extension at 72 °C for 30 sec. The number of PCR cycles was 30 for GAPDH and 35 for PLCε.

**Electrically-evoked Ca\(^{2+}\) Transients** - Electrically-evoked Ca\(^{2+}\) transients were measured as previously described (10). For each experiment, data were collected in the absence of agonist for 5-15 cells in order to determine naïve Ca\(^{2+}\) transient amplitude. 1 µM Isoproterenol and 10 µM cpTOME were prepared in control Ringer solution (145 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\) and 10 mM Hepes, pH=7.4) and locally perfused for 20 seconds followed by 60 seconds of electrical stimulation (20ms, 8V, 0.5-1 Hz, 60s) in the continued presence of agonist.

**Pharmacological Inhibition of PKC, IP\(_3\) Receptors and CamKII** – To determine the effect of PKC inhibition on electrically-evoked Ca\(^{2+}\) transient amplitude, cells were pre-treated for 5 minutes with 1 µM bis-indolylmaleimide-1 (BIM) (Calbiochem), a broad specificity PKC inhibitor, followed by constant perfusion of BIM in the presence of 1 µM Iso or 10 µM 8-4-(chlorophenylthio)-2'-O-methyladenosine-3',5'-monophosphate (cpTOME). For IP\(_3\) receptor inhibition, cells were pretreated with 20 µM 2-aminoethoxydihydroxybore (2-APB) for 5 minutes followed by constant perfusion of 2-APB in the presence of 1 µM Iso. For CamKII inhibition, cells were pretreated with 1 µM KN93 (or inactive KN92) for 30 minutes followed by constant perfusion of KN93 in the presence of 1 µM Iso or 10 µM cpTOME.

**PKC Translocation** AVM were isolated as described and plated at a density of 50,000 cells/60mm tissue culture dish in serum free MEM culture media supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin and 2.5 µM blebbistatin. After 2 hours, the media was changed to remove dead cells and debris. Cells were treated with 1 µM Iso for 30 seconds or 10 µM cpTOME for 3 minutes in a 37°C incubator. Following treatment, dishes were placed immediately on ice and media and agonist were removed. Cells were washed two times with ice-cold PBS supplemented with protease inhibitors. Cells were scraped into a lysis buffer (50 mM HEPES pH 8.0, 3 mM MgCl\(_2\), 100 µM EDTA, 100 mM NaCl, 50 µM NaVO\(_4\), and protease inhibitors) and probe sonicated. Samples were then centrifuged at 100,000g at 4°C for 15 minutes to pellet the membrane fraction. The membrane pellet was washed two times with ice cold lysis buffer, and then re-suspended in sample buffer (125 mM Tris-HCl pH 6.8, 20%
glycerol, 4% SDS, 10% β-mercaptoethanol (1.42 M), and 0.25% bromophenol blue. Samples were resolved by 10% SDS-PAGE and western blotted for specific PKC isoforms. PKCε and PKCα antibodies (Santa Cruz) were used at a 1:1000 dilution. HRP-conjugated anti-rabbit IgG (BioRad) was used at 1:10000.

PKC siRNA PKCε-specific and CY3-labelled negative control siRNAs (Ambion) were reconstituted at 100 pM. Wild type AVM were isolated as before and media was changed prior to transfection. For each siRNA, 600 pmol was added to 600 µL of OPTIMEM. In a separate tube, 6 µL of Lipofectamine 2000 was added to 600 µL of OPTIMEM. After 5 minutes, siRNA and Lipofectamine tubes were mixed and incubated at room temperature for 20 minutes. The 200 pmol siRNA mixture was then added to each 35mM dish of AVM. Efficiency of transfection was determined by fluorescence microscopy of AVM transfected with CY3, labelled negative control siRNA. Cells transfected with PKCε-specific siRNA or negative control siRNA were harvested in sample buffer at 24, 36, or 48 hours post-transfection. Knockdown of PKCε protein was determined by quantitative western blotting.

Western Blotting Phosphorylation Analysis Phospho-Thr286 CamKII (1:1000) and total CamKII specific (1:1000) antibodies were from Santa Cruz, Phospho-Thr17 PLB (1:6000), Phospho-Ser16 PLB (1:6000) and total PLB (1:6000) antibodies were from Badrilla. Phospho-Ser2815 Ryr2 antibody (1:2000) was kindly provided by Xander Wehrens, Baylor College of Medicine and total Ryr2 antibody (1:2000) was from Affinity Bioreagents.

Perforated Patch Clamp Briefly, individual AVM adhered to laminin-coated dishes were preloaded with 5 µM fluo-4 AM for 30 minutes at 37°C in a Ringer solution. Myocytes were then washed 2X with an external Ca2+ current recording solution containing: 140 mM TEACl, 2 mM MgCl2, 1.8 mM CaCl2, 0.005 mM blebbistatin, 10 mM glucose, and 10 mM Hepes, pH=7.4. Patch clamp electrodes had a pipette resistance of 1-2MOhm when backfilled with internal solution containing: 135 mM CsCl, 1 mM MgCl2, and 10 mM Hepes, pH=7.2, Ca2+ currents and transients were elicited using 200ms test pulses from -50 mV to +70 mV in 10 mV increments delivered at 10s intervals (0.1 Hz). Peak L-type Ca2+ current magnitude was normalized to total cell capacitance (pA/pF), plotted as a function of membrane potential (Vm), and fitted according to:

\[ I = G_{\text{max}} \left( V_m - V_{\text{rev}} \right) / \left( 1 + \exp \left[ \frac{\left( V_m - V_{G1/2} \right)}{k_G} \right] \right) \]

where \( G_{\text{max}} \) is the maximal L-channel conductance, \( V_m \) is the test potential, \( V_{G1/2} \) is the voltage of half-maximal activation of \( G_{\text{max}} \), \( V_{\text{rev}} \) is the extrapolated reversal potential, and \( k_G \) is a slope factor. The kinetics of Ca2+ current inactivation was described by fitting the inactivation phase to the following single exponential function:

\[ I(t) = A \exp \left( -t/\tau_{\text{inact}} \right) + C \]

where \( I(t) \) is the current at time \( t \) after the depolarization, \( A \) is the amplitude of the inactivating component of current, \( \tau_{\text{inact}} \) is the time constant of inactivation, and \( C \) represents the steady-state non-inactivating component of current. Ca2+ transients recorded during each test pulse were expressed as \( \Delta F/F \), where \( F \) represents baseline fluorescence and \( \Delta F \) represents the fluorescence change from baseline.
Rap and Ras Activation – Hearts were excised from 4-6 month old PLCε<sup>+/+</sup> or PLCε<sup>-/-</sup> mice, cannulated through the aorta, perfused in the presence or absence of 1 µM isoproterenol for 10 min, and snap frozen in liquid nitrogen. Heart lysates were prepared by polytron in a buffer containing 50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 2.5 mM MgCl<sub>2</sub>, 1% NP-40 in 10% glycerol, and protease inhibitors. 1 mg of heart lysate was incubated with GST-tagged fusion protein corresponding to amino acids 788-884 of human RalGDS, Rap, GTP binding domain or 1,149 of human Raf,1, Ras GTP binding domain bound to glutathione agarose in heart lysis buffer. Following incubation, beads were harvested by centrifugation, the supernatant removed, and beads were extensively washed with lysis buffer. After washing, beads were pelleted by centrifugation, resuspended in 4X SDS-sample buffer, resolved on a 15% polyacrylamide gel and transferred to nitrocellulose for western blotting.

Statistics - Data are given as mean ± standard error (SE). Statistical significance was determined using unpaired Student’s t-test and a one-way analysis of variance (ANOVA) for multiple comparisons. Differences were considered statistically significant at P < 0.05.

Results

To determine the relative roles of PLCε-RapGEF and PLC activities in the regulation of cardiac Ca<sup>2+</sup> handling, we transduced freshly isolated PLCε<sup>-/-</sup> myocytes with adenoviruses directing expression of either wild type PLCε or mutants of PLCε previously shown to eliminate either PLCε hydrolytic activity (PLCεH1460L)(3), RapGEF activity (PLCεΔCDC25), or Rap (and other small GTPases) binding to the RA2 domain (PLCεK2150E)(1) (Fig. 1A). Based on PCR analysis of PLCε transcripts, all constructs were expressed to similar levels 24 hours after transduction (Fig. 1B). Western blots of extracts from AVM infected with the PLCε mutant viruses indicate that the mutations do not affect expression of PLCε (Fig. 1C). Electrically-evoked Ca<sup>2+</sup> transients in transduced myocytes were then assessed in the presence or absence of either the βAR agonist isoproterenol (1 µM) or the direct Epac activator cpTOME (10 µM). As previously observed (12), isoproterenol-dependent enhancement of electrically-evoked Ca<sup>2+</sup> release was significantly increased 24 hours after transduction of wild type PLCε in PLCε<sup>-/-</sup> AVM (Fig. 1D). Wild type PLCε expression also restored the 2-fold increase in evoked Ca<sup>2+</sup> transient amplitude in response to Epac activation with cpTOME (Fig. 1E). In contrast, PLCε<sup>-/-</sup> AVM transduced with either PLCεH1460L or PLCεK2150E failed to respond to cpTOME and showed no increase in isoproterenol responsiveness compared to YFP control (Fig. 1C-E) (Supplemental Figure 1). These results confirm that direct stimulation of PLCε hydrolytic activity by binding of a Ras family GTPase (likely Rap1) to the PLCε RA2 domain is required for maximal βAR, mediated increases in electrically-evoked SR Ca<sup>2+</sup> release. PLCε<sup>-/-</sup> AVM transduced with PLCεΔCDC25, the RapGEF deficient PLCε mutant, failed to exhibit increased responsiveness to either isoproterenol or cpTOME (Fig. 1D,E) (Supplemental Figure 1), indicating that PLCε-RapGEF activity is also required for the proper execution of the Epac/PLCε pathway during βAR-mediated regulation of SR Ca<sup>2+</sup> release. We have previously shown that the Rap GEF deletion, PLCεΔCDC25, does not significantly affect intrinsic PIP<sub>2</sub> hydrolysis activity(9).

The inability of a RapGEF deficient mutant of PLCε to rescue cpTOME and maximal isoproterenol-stimulated enhancement of CICR in PLCε<sup>-/-</sup> AVM suggests that Rap activation downstream of βAR stimulation is at least partially dependent on
PLCε. Lysates prepared from control and iso-
perfused hearts of PLCε−/− or PLCε+/+ mice
were analyzed for activated Rap (RapGTP) by
pulldown with GST-RalGDS and activated
Ras (RasGTP) by pulldown with GST-Raf-1-
RBD. A significant increase in active Rap
over basal levels was observed in lysates from
Iso-treated PLCε+/+ myocytes (Fig. 2, left). In
lysates prepared from PLCε−/− mice, detectable
Rap activation was not observed under either
basal or Iso-treated conditions (Fig. 2, right).
On the other hand, similar levels of Ras
activation were observed under basal and Iso-
treated conditions in hearts from both PLCε+/+
and PLCε−/− mice. Total Rap and Ras levels
were identical between PLCε−/− and wild type
heart lysates. Together, these results are
consistent with previous in vitro findings that
PLCε acts specifically as a GEF for Rap, but
not Ras (2).

PLC-mediated hydrolysis of PIP$_2$
results in generation of IP$_3$ and diacylglycerol
(DAG) and the subsequent activation of Ca$^{2+}$
release through IP$_3$ receptors in the
sarcoplasmic reticulum and/or PKC activation,
respectively. A definitive role for IP$_3$-
mediated Ca$^{2+}$ release in EC coupling in
cardiac myocytes has not been identified
despite intensive investigation (14). Type 2
IP$_3$ receptors are the predominant IP$_3$R
isoform present in the heart. Analysis of type
2 IP$_3$R knockout mice indicates that the type 2
IP$_3$ receptor is not required for βAR
enhancement of Ca$^{2+}$ release in atrial cardiac
myocytes, but is important for endothelin
dependent regulation of Ca$^{2+}$ release (15). To
assess the potential role of IP$_3$ in our cells, we
compared electrically-evoked Ca$^{2+}$ release in
control, Iso-, and cpTOME-treated wild type
AVM after treatment with the IP$_3$ receptor
inhibitor, 2-APB (20 µM). 2-APB treatment
did not alter either the Iso (Fig. 3A) or
cpTOME (data not shown) responsiveness,
supporting conclusions of previous studies
that IP$_3$ receptors do not directly contribute to
Iso-dependent enhancement of CICR.

To test for PKC involvement in the
βAR and Epac responses, electrically-evoked
Ca$^{2+}$ transients in the presence or absence of
isoproterenol were compared in AVM pretreated with 1 µM bis-indolylmaleimide-1
(BIM), a broad-specificity PKC inhibitor.
BIM treatment significantly inhibited
isoproterenol-stimulated enhancement of SR-
Ca$^{2+}$ release in wild type AVM (Fig. 3B, left).
In addition, BIM treatment abolished the
increase in electrically-evoked Ca$^{2+}$ transient
amplitude observed following direct activation
of Epac with cpTOME (Fig. 3C). However,
BIM treatment did not alter Iso-stimulation of
electrically-evoked Ca$^{2+}$ transients in PLCε−/
myocytes (Fig. 3B, right) or baseline evoked
transients (data not shown). These results
indicate that the effects of BIM are specific to
the PLCε-dependent pathway downstream of
βAR stimulation and implicate PKC activation
in this pathway.

There are eleven distinct isoforms of
PKC, four of which are consistently detected
in cardiac myocytes: α, βII, δ, and ε (16). To
determine if a specific isoform of PKC is
activated downstream of PLCε, we monitored
translocation of specific PKC isoforms to the
particulate fraction following treatment of
freshly isolated PLCε+/+ and PLCε−/− cardiac
myocytes with either isoproterenol or
cpTOME. Western blot analysis of the
particulate fraction of wild type cardiac
myocytes revealed a specific increase in PKCε
in the membrane fraction in response to both
isoproterenol and cpTOME treatment relative
to non-treated control (Fig. 4A). In contrast,
PKCα did not translocate to the membrane
fraction in response to isoproterenol. In
PLCε+/− AVM, neither isoproterenol nor
cpTOME triggered translocation of PKCε to
the membrane (if anything a small decrease in
PKCε at the membrane was observed), placing
PKCε downstream of the βAR/Epac/Rap/PLCε pathway.

To further test the role of PKCε downstream of the Epac/Rap/PLCε pathway, wild type AVM were transfected with either PKCε-specific siRNA or a CY3-labelled negative control siRNA. Transfection efficiency was nearly one-hundred percent (Supplemental Figure 2) and western blot analysis revealed that PKCε (but not PKCα) protein levels were knocked down by at least 95% at 36 hours post-transfection (Fig. 4B). PKCε protein levels were not substantially decreased in cells transfected with the negative control siRNA at all time points monitored (24, 36, and 48 hours). Electrically-evoked Ca^{2+} transients were monitored 36 hours after transfection of wild type AVM with either PKCε or negative control siRNAs. Baseline electrically-evoked Ca^{2+} transients were not different between control and PKCε siRNA-treated AVM. On the other hand, peak Ca^{2+} transient amplitude in the presence of isoproterenol was significantly inhibited and cpTOME responses abolished (Fig. 4C) in AVM treated with PKCε siRNA. These data are consistent with results obtained following pharmacological PKC inhibition with BIM (Fig. 3B, C) and Iso,cpTOME-dependent PKCε membrane translocation (Fig. 4A), indicating that PKC acts downstream of PLCε and specifically implicates PKCε as the relevant PKC isoform involved.

A recent report demonstrated that CamKII is activated following Epac stimulation with cpTOME in rat cardiac myocytes, however the mechanism for CamKII activation by Epac was not determined (17). PKC has also been shown to activate CamKII in rat ventricular myocytes (18) and CamKII is directly phosphorylated at Thr286 by PKC in vitro (19). Therefore, we determined if CamKII activation is required for Iso- and Epac-dependent enhancement of CICR, and if it is downstream of PKC. Iso- and cpTOME-induced enhancement of electrically-evoked Ca^{2+} transients in wild type AVM were determined in the absence and presence of KN93, a specific CamKII inhibitor. KN93, but not the control compound KN92, attenuated Iso-induced enhancement of evoked release (Fig. 5A) and completely blocked cpTOME-induced enhancement (Fig. 5B). Co-treatment with BIM and KN93 did not further diminish the response to isoproterenol relative to treatment with either compound alone (data not shown). Additionally, KN93 had no effect on the Iso response in PLCε−/− AVM (Fig. 5A) or on baseline evoked transients (data not shown), supporting specific involvement of CamKII in the PLCε dependent pathway.

To determine if CamKII activation was dependent on PKC, wild type cardiac myocytes were treated with either isoproterenol or cpTOME alone or in the presence of BIM and CamKII phosphorylation at Thr286 was measured by western blotting (Fig. 5C). Both isoproterenol and cpTOME treatment increased CamKII phosphorylation at Thr286 relative to non-treated control. The cpTOME-dependent increase was blocked in the presence of BIM and the Iso-dependent increase was partially blocked by BIM. These data support the conclusion that the Epac/PLCε pathway can control CamKII activation in a PKCε-dependent, Ca^{2+}-independent manner. That the Iso-dependent increase in phosphorylation was only partially blocked suggests that there are multiple mechanisms for CamKII activation downstream of Iso, one of which includes the Ca^{2+}-independent Epac and PKC pathway, but may also result from changes in Ca^{2+} that occur with Iso-dependent regulation of PKA.

CamKII phosphorylates numerous Ca^{2+}-handling proteins, including the L-type
Ca\textsuperscript{2+} channel, Ryr2 and PLB, involved in precisely controlling dynamic changes in intracellular calcium levels during the cardiac cycle (20). CamKII-dependent modulation of RyR function by phosphorylation at Ser2815 has been implicated as a means for positive regulation of SR-Ca\textsuperscript{2+} release downstream of βAR stimulation. CamKII also phosphorylates PLB at Thr17 to stimulate SR Ca\textsuperscript{2+} reuptake to increase content available for release. To determine if Epac/PLC\textgreek{e}-stimulation results in a PKC-dependent, CamKII-mediated, phosphorylation of Ryr2 and/or PLB, AVM were treated with cpTOME in the presence or absence of the PKC inhibitor BIM. Phosphorylation at the CamKII specific sites was measured by western blotting (Fig. 5D). cpTOME treatment significantly enhanced phosphorylation of Ryr2 at Ser2815 and PLB at Thr17 in wild type AVM. These increases were ablated in AVM pretreated with BIM. These data identify at least two effector targets (Ryr2 and PLB) of the Epac/PLC\textgreek{e} pathway that could be involved in regulating the magnitude of CICR.

To examine the relationship between Ca\textsuperscript{2+} influx and release during EC coupling we conducted voltage clamp experiments to determine if the Epac/PLC\textgreek{e} pathway alters the properties of depolarization-induced L-type Ca\textsuperscript{2+} channel function and RyR2-mediated Ca\textsuperscript{2+} release. Perforated patch clamp experiments in fluo-4-loaded AVM were conducted to simultaneously compare the voltage dependence, magnitude, and kinetics of L-type Ca\textsuperscript{2+} currents and global intracellular Ca\textsuperscript{2+} transients in wild type and PLC\textgreek{e}\textsuperscript{-/-} myocytes before and after βAR activation (Fig. 6 and Supplemental Figure 3). L-type Ca\textsuperscript{2+} currents and intracellular Ca\textsuperscript{2+} transients were elicited by 200 ms test pulses from -50 to +70mV at 10mV increments. No differences in the magnitude or kinetics of L-type Ca\textsuperscript{2+} currents (Fig. 6A) or global intracellular Ca\textsuperscript{2+} transients (Fig. 6C) were observed between PLC\textgreek{e}\textsuperscript{-/-} and PLC\textgreek{e}\textsuperscript{+/-} AVM under basal conditions (closed symbols) (Table 1). Application of 1 μM Iso (open symbols) caused a similar 1.5-2 fold increase in peak L-type Ca\textsuperscript{2+} current density (Fig. 6A) and maximum channel conductance (Fig. 6B and Table 1) in both PLC\textgreek{e}\textsuperscript{-/-} and PLC\textgreek{e}\textsuperscript{+/-} AVM. However, peak Iso-stimulated Ca\textsuperscript{2+} transient amplitude was significantly attenuated in PLC\textgreek{e}\textsuperscript{-/-} cardiac myocytes (Fig. 6C and D and Table 1), consistent with results observed in intact myocytes (Fig. 3B and 5A). In addition, the kinetics of Ca\textsuperscript{2+} current inactivation was significantly slower in myocytes from PLC\textgreek{e}\textsuperscript{-/-} mice (Supplemental Figure 3). These data indicate that alterations in action potential or L-type channel activity are not necessary for PLC\textgreek{e}-dependent regulation of CICR.

**Discussion**

PLC\textgreek{e} is unique among PLC enzymes in that it possesses both phospholipase C and RapGEF activities. Physiological roles for both catalytic functions of PLC\textgreek{e} are beginning to emerge (9;10;21;22). Here we demonstrate that both PLC\textgreek{e} hydrolytic and RapGEF activities are required for maximal βAR-mediated (and Epac-dependent) enhancement of electrically-evoked Ca\textsuperscript{2+} release in the heart. We hypothesize that the PLC\textgreek{e},RapGEF activity ensures sufficient Rap activation to maintain PLC\textgreek{e} hydrolytic activity, and that PLC\textgreek{e} is required for sustained Rap activation in the heart. Epac stimulation by cAMP may initiate a low level of Rap activation that (undetectable in the PLC\textgreek{e}\textsuperscript{-/-} myocytes), in turn, stimulates PLC\textgreek{e} to significantly amplify Rap activation that feeds forward to further stimulate PLC\textgreek{e} and subsequent regulation of CICR. This model is supported by previous studies in transfected cells demonstrating that PLC\textgreek{e} potentiates its own activation by RapGTP (2) and in primary
astrocytes where PLCε RapGEF activity is required for sustained Rap activation and downstream ERK signaling (9). In addition, it is important to note that Rap-GTP generated from PLCε may also regulate other enzymes in the heart such as ERK5 where Rap-mediated inhibition protects against the development of hypertrophy (23). This would be consistent with our findings that PLCε−/− mice exhibit increased susceptibility to stress induced hypertrophy (10) and that PLCε RapGEF activity modulates ERK signaling in astrocytes(9).

Downstream of PLCε activity, we demonstrate that diacylglycerol-mediated PKCε activation is required for maximal βAR-dependent enhancement of CICR (Fig. 3). Several reports have suggested that PKC activity modulates CICR in cardiac myocytes. Treatment with norepinephrine or phorbol myristic acid causes PKCε translocation to cross-striated t-tubular regions of cardiac myocytes upon activation, strategically placing this enzyme in position to phosphorylate proteins involved in Ca2+ handling (24). PKCδ and PKCε have been shown to mediate positive inotropy that is dependent on subcellular localization (25). Our data are consistent with a positive ionotropic effect of PKCε and indicate that PLCε is an upstream regulator of PKCε in the heart. siRNA knockdown of PKCε strongly suppressed cpTOME-dependent increases in Ca2+ transient amplitudes suggesting that PKCε is the major isoform of PKC involved in Epac/PLCε-dependent responses. On the other hand, the inhibition of Iso-dependent responses by PKCε siRNA appeared less than with observed with BIM treatment (Fig. 3B vs. 4C). One possibility is that another PKC isoform such as PKCδ is involved in the Iso-dependent response or that a more complete inhibition of PKCε that might be achievable with BIM may be required to fully inhibit the Iso-response.

We also identified PKC-dependent CamKII regulation as an essential downstream component of the Epac/PLCε pathway in AVM. βAR- and cpTOME enhancement of electrically-evoked Ca2+ release was suppressed by PKC inhibition with BIM (Fig. 3B, C), PKCε knockdown (Fig. 4C), and CamKII inhibition with KN93 (Fig. 5) in PLCε+/− AVM. On the other hand, neither PKC inhibition, CamKII inhibition, nor the combination, had any significant effect on the already reduced Iso-dependent regulation of evoked release in PLCε−/− AVM, indicating that both PKCε and CamKII are downstream from PLCε activation. We also show that phosphorylation of CamKII at Thr286 is increased by cpTOME in a PKC-dependent manner. These data are consistent with a previous report demonstrating that activation of Epac stimulates CamKII Thr286 phosphorylation (17) but extend this result to show that Epac-dependent CamKII activation relies on PLCε-dependent PKCε activity. While a mechanism for PKC-dependent activation of CamKII has not been clearly delineated, CamKII Thr 286 has been shown to be directly phosphorylated by PKC in vitro (19). Thr286 autophosphorylation results in tight binding of calmodulin such that Ca2+ is no longer required for activation. If CamKII Thr286 is directly phosphorylated by PKC it should also result in Ca2+-independent regulation. Physiological evidence for PKC-dependent regulation of CamKII is sparse but two previous studies have shown that α-adrenergic receptor stimulation facilitates PKC-mediated activation of CamKII (18;26). PKCε may not directly phosphorylate CamKII in cardiac myocytes in response to Epac/PLCε activation but we clearly demonstrate that PKC activation is upstream of CamKII Thr286 phosphorylation in this pathway. It is also possible that PKCε itself phosphorylates
components of the Ca\(^{2+}\) handling machinery, but our data indicate that any such activity alone is insufficient since CamKII inhibition with KN93 completely blocks cpTOME dependent enhancement of Ca\(^{2+}\) transient amplitudes (Fig. 5B). This indicates that PKC activation by this pathway is not sufficient to cause increases in Ca\(^{2+}\) transients. Nevertheless, it remains formally possible that local Ca\(^{2+}\) release, dependent on PKC activation, could lead to CamKII autophosphorylation. We propose that in cardiac myocytes, linear activation of Epac-PLCe-PKCe-CamKII mediates a component of Iso-dependent regulation of evoked Ca\(^{2+}\) release in cardiac myocytes.

Potential targets downstream of this pathway include the L-type Ca\(^{2+}\) channel, Ryr2 and phospholamban. PLC\(\varepsilon\) ablation did not markedly affect isoproterenol-stimulated increases in L-type Ca\(^{2+}\) channel current density in perforated patch clamp experiments (Fig. 6A, B) and L-type Ca\(^{2+}\) channel activity was not significantly altered by 20 \(\mu\)M cpTOME (data not shown). In the same cells Iso-stimulated enhancement of depolarization-induced Ca\(^{2+}\) transients was significantly attenuated (Fig. 6C, D). The fact that Ca\(^{2+}\) release elicited by a uniform voltage clamp pulse is reduced in PLC\(\varepsilon\)-myocytes, suggests that changes in action potential waveform are not likely responsible for the reduction in Iso responsiveness. Together, these data indicate that the Epac/PLCe/PKCe/CamKII pathway contributes to enhanced release of Ca\(^{2+}\) from the SR during \(\beta\)AR-stimulation that is not dependent on changes in Ca\(^{2+}\) influx. Two proteins that control release of Ca\(^{2+}\) from the SR, Ryr2 and PLB, are phosphorylated at CamKII-specific sites in response to Epac stimulation, supporting this idea. The observed increase in Ca\(^{2+}\) release in response to a uniform Ca\(^{2+}\) influx could arise from a combination of both an increase in Ryr2 sensitivity to activation by Ca\(^{2+}\) influx(27;28) and an increase in Ca\(^{2+}\) reuptake and load. The observed increase in Ca\(^{2+}\) release we report here differs from results of Pereira et al.(17) who report that Epac activation decreases evoked Ca\(^{2+}\) release due to CamKII dependent SR store depletion. Apparent discrepancies between our results and Pereira et al. could be due to differences in protocol or species used, but the overall conclusions that Epac activation results in CamKII activation and Ryr2(S2815) phosphorylation are in agreement.

Roles of PKC and CamKII in both normal and pathological cardiac function have been steadily emerging over the last several years, but our understanding of the physiological mechanisms that control activation of these pathways have lagged behind. Here, we have outlined a novel, PLC\(\varepsilon\)/PKCe/CamKII-dependent regulatory mechanism for regulating cardiac CICR in adult ventricular cardiac myocytes. Previous studies have implicated CamKII-dependent phosphorylation of RyR2 as a means for regulating SR-Ca\(^{2+}\) release downstream of Epac or \(\beta\)AR stimulation (17;28). We extend these findings by identifying key mechanistic links between Epac activation and regulation of CICR such that the majority of the components of the pathway are now defined. This pathway is clearly important for cardiac function because mice lacking PLC\(\varepsilon\) exhibit a significantly impaired ability to respond to \(\beta\)AR-stimulation. This impairment is manifested by both decreased cardiac function in response to isoproterenol administration in whole animals (10) and decreased \(\beta\)AR-dependent enhancement of CICR in AVM isolated from PLC\(\varepsilon\) mice (12). PLC\(\varepsilon\) mice also show an increased sensitivity to stress induced cardiac hypertrophy (10) and it remains to be determined how components of the pathway could contribute to this pathology.
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Footnotes:

**Abbreviations:** PLC, Phosphoinositide-specific phospholipase C; PIP\(_2\), Phosphatidylinositol 4,5 bisphosphate; IP\(_3\), inositol 1,4,5 trisphosphate; GEF, guanine nucleotide exchange factor; βAR, β-adrenergic receptor; AVM, adult ventricular myocytes; PKA, protein kinase A; PKC, protein kinase C; CICR, calcium-induced calcium release; SR, sarcoplasmic reticulum; PLB, phospholamban; Ryr, ryanodine receptor; BIM, bisindolylmaleimide-1; cpTOME, 8-4-(chlorophenylthio)-2′-O-methyladenosine-3′,5′-monophosphate.

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Figure 1. Epac-mediated enhancement of CICR requires both PLCε hydrolytic and RapGEF activities. (A), Domain structure of PLCε (CDC25 GEF – Ras family small GTPase guanine nucleotide exchange factor domain, PH – pleckstrin homology, EF – EF-hand Ca2+-binding domain, X and Y – PIP₂ hydrolysis catalytic domain, C2 – Ca2+ dependent lipid-binding domain, RA1 and RA2 – Ras association domains). ∆CDC25(677,772) – GEF deletion mutant, no RapGEF activity, H1460L – catalytic domain point mutation, lacks PIP₂ hydrolysis activity, K2150E – RA2 domain point mutation – eliminates stimulation of PLC activity by Ras and Rap1. (B) PLCε−/− cardiac myocytes were transduced with YFP, PLCε wild type, or PLCε domain mutant adenoviruses. 24 hours post-transduction, equal expression of PLCε mRNA was demonstrated by RT-PCR (lower). (C) Cardiac myocytes were transduced with wt and mutant PLCε viruses and protein expression was measured after 48h by Western blotting (D, E). Average (±S.E.) peak Ca2+ transient amplitude (Δ405/485) for naïve PLCε−/− myocytes and 24 hours post-transduction with either YFP control, PLCε wild type or PLCε domain mutant adenovirus (300 m.o.i.) with or without 1µM isoproterenol (D) or 10µM cpTOME (E).

Figure 2. PLCε is required for βAR-mediated Rap activation in cardiac myocytes. Hearts from PLCε+/+ or PLCε−/− mice were cannulated through the aorta and perfused with or without 1µM isoproterenol for 10 min. 1mg of heart lysate was incubated with either GST-tagged RalGDS-RBD for assaying activated Rap or GST-tagged Raf1-RBD for activated Ras. Gβ1 was used as a gel loading control. Data are representative of 3 experiments showing similar results.

Figure 3. Pharmacological inhibition of PKC attenuates βAR enhancement of CICR. (A), IP₃R inhibition with 2-APB (20 µM, 5 min pre-treatment) does not inhibit βAR-dependent increases in Ca2+ transient amplitude (Δ405/485). Ca2+ transients were measured in the absence and presence of 1µM isoproterenol. Data are pooled from 10-15 cells per treatment condition. Results are average (±S.E.). (B and C), PKC inhibition (1µM bis-indolylmaleimide (BIM), 5 minute pre-treatment) significantly blunts (B) isoproterenol - and (C) cpTOME -induced increases in Ca2+-transient amplitude in PLCε+/+, but not PLCε−/− cardiac myocytes. BIM did not affect naïve Ca2+ transient amplitude. Data were pooled from 20-40 cells for each treatment condition from n = 3 PLCε+/+ and n = 2 PLCε−/− mice. Results are average (±S.E.); ***, p<0.001; #, p<0.001 for PLCε−/− response compared to PLCε+/+; ns is not significant; one-way ANOVA, Bonferroni post test.

Figure 4. PLCε-dependent enhancement of CICR requires specific activation of PKCε. (A), left - PKCε translocates to the membrane fraction following treatment with 1µM isoproterenol (30 sec) or 10µM cpTOME (3 min) in PLCε+/+ but not PLCε−/− cardiac myocytes. PKCα does not translocate to the membrane in response to βAR stimulation. PMA treatment (500nM, 10 min.) was used as a positive control for PKC translocation. 3 µg of cardiac myocyte membrane fractions was analyzed for PKC isoform translocation. Gβ subunit was used as a loading control. right - Densitometric quantitation of PKCε membrane translocation from cells isolated from 5 PLCε+/+ and 3 PLCε−/− mice. Data are represented as a percentage of maximal translocation evoked by PMA treatment. **, p<0.01, ***p<0.001, ns – not significant as
compared to nontreated PLCε+/+ cells. One-way ANOVA, Bonferroni post-test. (B), left - PLCε+/+ cardiac myocytes were transfected with PKCε-specific siRNA or a CY3-labelled negative control siRNA. PKCε protein levels are nearly completely knocked down in cardiac myocytes transfected with PKCε-specific siRNA relative to negative control siRNA at 36 hours post-transfection. Lower left – PKCα protein levels are not significantly affected by PKCε siRNA 36 hours post-transfection. Right - Densitometric quantitation of PKCε protein expression from myocytes transfected with either PKCε siRNA or CY3-labeled negative control siRNA pooled from three separate experiments (C), Knockdown of PKCε significantly decreases isoproterenol-induced enhancement of CICR and completely eliminates cpTOME responsiveness in PLCε+/+ cardiac myocytes. Data are pooled Ca²⁺ transient amplitudes (Δ404/485) from 20-40 cells per condition, n = 3 mice. Results are average (±S.E.); ***, p<0.001, one way ANOVA, Bonferroni post-test.

Figure 5. PKC-dependent activation of CamKII is required for PLCε-mediated enhancement of CICR CamKII inhibition (1µM KN-93, 30 minute pre-treatment) significantly blunts (A) Iso- and (B) cpTOME (10µM) enhancement of electrically-evoked Ca²⁺ transient amplitude in AVM from PLCε+/+, but not PLCε−/− mice. Pretreatment with KN92, an inactive analogue of KN-93, does not affect Iso responsiveness. Data were pooled from 5-15 cells for each treatment condition from 3 PLCε+/+ and 3 PLCε−/− mice. Results are average (±S.E.); ***, p<0.001; ns – not significant; one-way ANOVA, Bonferroni post test. (C) Phosphorylation of CamKII at Thr286 was measured by Western blotting of extracts of AVM treated with Iso (1µM), cpTOME (10µM) and BIM (1µM) as indicated. (n=5 animals). right panel pooled data from densitometric quantitation. Results are average (±S.E.); *, p<0.05; one-way ANOVA, Bonferroni post test. (D) Phosphorylation of Ryr2 S2815 and PLB T17 was measured by Western blotting of extracts of AVM treated with cpTOME (10µM) or BIM (1µM) as indicated (n=3 animals each). right panel pooled data from densitometric quantitation and normalized relative to untreated cells. Results are average (±S.E.); **, p<0.01, ***, p<0.001; one-way ANOVA, Bonferroni post test.

Figure 6. PLCε ablation significantly reduces isoproterenol stimulation of depolarization-induced intracellular calcium transients without altering L-type Ca²⁺ current density. Perforated patch clamp experiments were used to simultaneously monitor depolarization-induced L-type Ca²⁺ currents (A, B) and intracellular Ca²⁺ transients (C, D) in the absence (closed symbols) and presence (open symbols) of βAR-stimulation with 1µM isoproterenol in AVM from PLCε+/+ (circles) and PLCε−/− (squares) mice. A, Average (±SE) voltage dependence of L-type Ca²⁺ current density. B, Average (±SE) fold stimulation of maximal L-type Ca²⁺ conductance in AVM from 5 PLCε−/− and 5 PLCε+/+ mice. C, Average (±SE) voltage dependence of intracellular Ca²⁺ transients. D, Average (±SE) fold stimulation of peak intracellular Ca²⁺ transient (measured at -30 mV) in AVM from 5 PLCε−/− and 5 PLCε+/+ mice. * p<0.05, t test.
Table 1. Parameters of IV and (∆F/F)$_{\text{max}}$ Data

|                   | $G_{\text{max}}$ (nS/nF) | $k_v$ (mV) | $V_{G1/2}$ (mV) | $V_{\text{rev}}$ (mV) | (∆F/F)$_{\text{max}}$ |
|-------------------|---------------------------|------------|-----------------|-----------------------|-----------------------|
| PLCε$^{+/+}$ Naïve (n=5 mice) | 108±14 | 7.4±0.4 | -14.3±0.4 | 62.9±1.1 | 0.15±0.04 |
| PLCε$^{+/+}$ Iso (n=5 mice) | 197±14* | 6.1±1.4 | -18.9±1.7 | 65.0±0.9 | 0.45±0.04*# |
| PLCε$^{-/-}$ Naïve (n=5 mice) | 103±14 | 6.6±0.3 | -16.0±2.4 | 64.7±1.6 | 0.15±0.03 |
| PLCε$^{-/-}$ Iso (n=5 mice) | 162±18** | 6.0±0.2 | -20.1±2.0 | 66.2±1.9 | 0.27±0.04** |

Table 1. Values represent mean (±SE) for $n$ number of mice (from a total of 32 PLC$^{+/+}$ and 27 PLC$^{-/-}$ myocytes). Parameters for the voltage dependence of Ca$^{2+}$ current ($I$-$V$) was obtained by fitting myocytes within each group separately to the appropriate equation ($I$-$V$, Eq. 1) as described in Methods. (∆F/F)$_{\text{max}}$ values are the mean (±SE) values obtained at a test potential of -10 mV. $G_{\text{max}}$, maximal L-channel conductance; $V_{\text{rev}}$, reversal potential; $V_{G1/2}$, potential at which $G$ is half-maximal, respectively; $k_v$ slope factor for $I$-$V$. *p<0.01 compared to PLCε$^{+/+}$ naive. **p<0.05 compared to PLCε$^{-/-}$ naive. #p<0.05 compared to PLCε$^{-/-}$ Iso.
Figure 1
Figure 2
Figure 3
Figure 4

A

| PLCε⁺/+ | PLCε⁻/- |
|---|---|
| NT | ISO | cpTOME | PMA | NT | ISO | cpTOME | PMA |

PKCε

Gβ

PKCα

Gβ

PLCε⁺/+  

B

| PKCεsiRNA | Control siRNA |
|---|---|
| 0 24 36 48 | 0 24 36 48 |

t(hr)

PKCε

Gβ

PKCεsiRNA

0 36 t(hr)

PKCα

Gβ

PKCεsiRNA

C

1μM ISO  
10μM cpTOME

ΔA0540/85

Ctrl PKCε ⋆⋆⋆
Figure 5
EPAC and phospholipase C regulate Ca\(^{2+}\) release in the heart by activation of protein kinase C and calcium-calmodulin-kinase II.

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