Phospholipase Cε Scaffolds to Muscle-specific A Kinase Anchoring Protein (mAKAPβ) and Integrates Multiple Hypertrophic Stimuli in Cardiac Myocytes*‡

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Lianghui Zhang1, Sundeep Malik1, Grant G. Kelley1, Michael S. Kapiloff1, and Alan V. Smrcka1,2

From the 1Department of Pharmacology and Physiology and the 2Aab Institute of Cardiovascular Science, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642, the 3Department of Hospital Medicine, Saint Joseph’s Hospital Health Center, Syracuse, New York 13203, and the 4Departments of Medicine and Pediatrics, Interdisciplinary Stem Cell Institute, University of Miami Miller School of Medicine, Miami, Florida 33101

To define a role for phospholipase Cε (PLCe) signaling in cardiac myocyte hypertrophic growth, PLCe protein was depleted from neonatal rat ventricular myocytes (NRVMs) using siRNA. NRVMs with PLCe depletion were stimulated with endothelin (ET-1), norepinephrine, insulin-like growth factor-1 (IGF-1), or isoproterenol and assessed for development of hypertrophy. PLCe depletion dramatically reduced hypertrophic growth and gene expression induced by all agonists tested. PLCe catalytic activity was required for hypertrophy development, yet PLCe depletion did not reduce global agonist-stimulated inositol phosphate production, suggesting a requirement for localized PLCe activity. PLCe was found to be scaffolded to a muscle-specific A kinase anchoring protein (mAKAPβ) in heart and NRVMs, and mAKAPβ localizes to the nuclear envelope in NRVMs. PLCε−mAKAPβ interaction domains were defined and overexpressed to disrupt endogenous mAKAPβ−PLCe complexes in NRVMs, resulting in significantly reduced ET-1−dependent NRVM hypertrophy. We propose that PLCe integrates multiple upstream signaling pathways to generate local signals at the nucleus that regulate hypertrophy.

Pathologic hypertrophic growth of the myocardium, a process associated with the development of heart failure, occurs in response to various stimuli including pressure overload and chronic increases in circulating hormones that place long term stress on the heart. Many of these hormones act on receptors in cardiac myocytes including adrenergic and endothelin receptors to initiate hypertrophic signaling cascades (1–3). The exact participants in these hypertrophic signaling cascades have been the subject of intense investigation. One common signaling mechanism closely coupled to receptor activation is activation of phosphatidylinositol-specific phospholipase C (PLC)4 (4). A role for PLC activity in regulation of hypertrophic signaling has been suggested because G_q, a potent stimulator of hypertrophy (5), directly stimulates PLCβ as a major regulatory pathway (6). Additionally, growth factors such as insulin-like growth factor-1 (IGF-1) that stimulate physiological hypertrophy activate PLCγ (7, 8). Recently, it has been reported that a splice variant of PLCβ, PLCβ1b, is required for hypertrophy downstream of α1-adrenergic receptor stimulation and suggested to be a major mediator of G_q-dependent cardiac hypertrophy (9).

The PLC family consists of 12 isoforms regulated by diverse upstream signal inputs, all of which hydrolyze phosphatidylinositol 4,5-bisphosphate to diacylglycerol (DAG) and inositol trisphosphate (IP3) (10, 11). Although containing a common PLC catalytic core domain, PLCβ, PLCγ, PLCδ, and PLCe isoforms are very different in terms of overall domain structure and regulatory mechanisms, indicating that they are unlikely to play overlapping roles in the cells. PLCe is unique in that it is directly regulated by various small GTPases, including Ras, Rho, and Rap (12–16), and Gβγ subunits, but not Gαq. PLCe has been shown to signal downstream of G protein-coupled receptors, including β-adrenergic receptors (BARs), lysophosphatidic acid and sphingosine 1-phosphate receptors (EdgRs), protease-activated receptors (13, 17), and growth factor receptor tyrosine kinases, including PDGF receptors (15) and EGF receptors (13). Thus, PLCe is ideally positioned to integrate multiple signaling inputs in various cell types including cardiac myocytes.

Recent studies of PLCe−/− mice revealed alterations in βAR-dependent regulation of cardiac ionotropy and hypertrophy (18). These mice had no hypertrophy at baseline but developed greater hypertrophy than wild type animals in response to chronic isoproterenol treatment. Thus, PLCe appeared to protect mice from stimulus-induced cardiac hypertrophy. In wild type mice, the levels of PLCe mRNA and protein increase during pressure overload or chronic isoproterenol (Iso) treatment. PLCe RNA levels are elevated in biopsies from human heart failure samples. To explore a mechanistic role for PLCe in hypertrophy, we utilized an siRNA protocol in an established cellular model of cardiac hypertrophy, the neonatal rat ventricu-
ular myocyte (NRVM) (19). Surprisingly, in this cell model, we found that PLCε appears to be required for development of cardiac hypertrophy downstream of multiple hypertrophic stimuli, rather than protective.

We also examined the subcellular scaffolding of PLCε that might lead to generation of IP₃ and DAG at specific cellular sites critical for activation of hypertrophic transcriptional mechanisms. A common family of scaffolding molecules in the heart is the protein kinase A anchoring protein (AKAP) family (20, 21). One member of this family that is involved in cytokine- and adrenergic-induced hypertrophy is the alternatively spliced isoform of muscle AKAP (mAKAP) expressed exclusively in striated myocytes (22). mAKAP binds multiple proteins besides protein kinase A (PKA) including exchange factor activated by cAMP (Epac), adenylate cyclase 5 (23), cAMP-phosphodiesterase 4D3 (PDE4D3), extracellular signal-regulated kinase 5 (ERK5) (22), protein phosphatase 2A (PP2A) (21), and calcineurin (24). mAKAP localizes to the nuclear envelope of myocytes, where it is targeted by nesprin-1α (25). It has also been suggested that mAKAP scaffolds PKA to ryanodine receptor type 2 (RyR2) in the sarcoplasmic reticulum (SR) (26), but this is controversial. Here we demonstrate that PLCε binds directly to mAKAP and that the scaffolding by mAKAP is important for the ability of PLCε to regulate cardiomyocyte hypertrophy. This suggests that scaffolding of PLCε to the nuclear envelope by mAKAP regulates expression of hypertrophic genes.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—mAKAP expression vectors are as described previously (25). All mAKAP vectors are numbered according to mAKAP because mAKAP is identical to mAKAPα 245–2314 (27). PLCε RA1 and PLCε RA2 were in pFLAG3. PLCε ACT and PLCε ΔGEF were expressed under the CMV promoter in pCMVscript.

**Adenoviral Constructs**—Short interfering hairpin RNAs (siRNAs) were designed for targeting mouse, rat, or human PLCε: siRNAPlcε 5′ GCCAATTACCTACAGGA; RANsiRNA, ACTGTCACAAGTACCTACA (scrambled siRNAPlcε 5′). siRNA sequences were subcloned into pShuttle-CMV as in (28), and recombinant viruses were generated following the manufacturer’s protocol for the Q-biogene AdEasy adenoviral vector system. Adenoviruses expressing PLCε-RA1 and mAKAP-SR1 were prepared by PCR amplification of the appropriate cDNA fragments and were subcloned into a shuttle vector, under the control of the mouse cytomegaloavirus promoter, which also expresses the YFP protein under the control of a separate mouse cytomegaloavirus promoter. The shuttle vector was recombined with the parent vector in HEK293 cells to generate adenovirus expressing the PLCε-RA1 or mAKAP-SR1 domain. Recombined virus was amplified in HEK293 cells and purified by CsCl gradient centrifugation. Viral titers were calculated by an immunoreactivity spot assay (29).

**Glutathione S-Transferase (GST) Fusion Proteins**—GST fusion protein constructs were prepared as described previously (23). BL21 DE3 cells containing pGEX-4T2 plasmids with the indicated regions of mAKAP fused to the C terminus of GST were grown to an optical density (600 nm) of 0.6 followed by induction with 100 μmol/liter isopropyl-1-thio-β-D-galactopyranoside for 16 h at 18 °C in 1 liter of each. Cells were harvested by centrifugation followed by lysis in 30 ml of lysis buffer (10 mmol/liter Tris-Cl, pH 7.5, 150 mmol/liter NaCl, 2 mmol/liter EDTA, and 10 mmol/liter β-mercaptoethanol) + protease inhibitors: 133 μM phenylmethanesulfonyl fluoride, 21 μg/ml 1-chloro-3-lysylamide-7-amino-2-heptanone and 1-lysylamide-2-phenylethyl chloromethyl ketone, 0.5 μg/ml aprotonin, 0.2 μg/ml leupeptin, 1 μg/ml pepstatin A, 42 μg/ml p-tosyl-l-arginine methyl ester, 10 μg/ml soybean trypsin inhibitor, by probe sonication. Nonidet P-40 detergent was added to a final concentration of 1% (v/v), and the samples were incubated on ice for 30 min. Lysates were cleared of insoluble material by centrifugation at 100,000 × g for 30 min. Soluble lysates were incubated with 500 μl of glutathione agarose beads (Thermo Fisher) for 2 h at 4 °C. Beads were harvested by centrifugation and washed four times in 20 ml of lysis buffer. Beads bound to GST fragments were analyzed directly by SDS-PAGE and staining with Coomassie Blue as well as by an Amido Black protein assay and stored at 4 °C.

For GST-mAKAP fragment pulldowns, 2 μg of GST fusion protein bound to 25 μl of beads was mixed with 50 ng of purified PLCε in 500 μl of pulldown buffer (10 mmol/liter Tris-Cl, pH 7.5, 150 mmol/liter NaCl, 2 mmol/liter EDTA+) . Mixtures were rotated for 2 h at 4 °C. Beads were harvested by centrifugation at 16,000 × g at 4 °C for 2 min. After supernatant removal, beads were washed three times with 500 μl of pull-down buffer. Beads were boiled in 30 μl of sample buffer, and 10 μl was loaded on a 7.5% (w/v) SDS-polyacrylamide gel for resolving and Western blotting.

**PLCε Purification**—His₅-tagged PLCε was purified according to previously published procedures (12).

**HEK Cell Culture and Transfection**—5 × 10⁵ HEK293 cells cultured in Dulbecco’s modified Eagle’s medium (DMEM) + 10% (v/v) FBS were plated on poly-D-lysine-coated 6-well plates the day before transfection. 2 μg of DNA was transfected using Lipofectamine Plus reagent (Invitrogen). Proteins were expressed for 48 h prior to harvesting for immunoprecipitation.

**Immunoprecipitation, Immunocytochemistry, and Western Blotting**—Cells were lysed in 1% (v/v) Nonidet P-40 lysis buffer (10 mmol/liter Tris-Cl, pH 7.5, 50 mmol/liter NaCl, 30 mmol/liter sodium pyrophosphate, 50 mmol/liter NaF, 100 μmol/liter phenylmethanesulfonyl fluoride, and 1% (v/v) Nonidet P-40). After sonication and centrifugation, the supernatant was incubated overnight with anti-Myc antibody (Covance) and Protein A/G agarose beads (Rockfort, CA) at 4 °C with rocking. Beads were centrifuged for 1 min at 16,000 × g, washed twice with 1.0 ml of lysis buffer, washed once with 1.0 ml of phosphate-buffered saline, boiled in 50 μl of 2× SDS sample buffer, and loaded onto a 7.5% (w/v) SDS-polyacrylamide gel. After SDS-PAGE, proteins were transferred to nitrocellulose for 16 h at 25 V followed by immunoblotting for mAKAP (anti-Myc antibody) or PLCε.

**Neonatal Cardiac Myocyte Culture, Adenoviral Infection, and Hormone Treatment**—NRVMs were isolated and cultured from hearts from 2–3-day-old Sprague-Dawley rats as has been previously described (19) with modifications. Briefly, hearts
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were excised, and ventricles were separated and rinsed in Hanks’ balanced salt solution prior to digestion with three rounds of collagenase type II (Worthington) in Hanks’ balanced salt solution without Ca\(^{2+}\) and Mg\(^{2+}\). Cells were collected by centrifugation and resuspended in DMEM containing 10% (v/v) fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mmol/liter glutamine, and 2 μg/ml vitamin B-12. Non-myocytes were removed by preplating of the cells for 1 h at 37 °C. The NRVMs were cultured in DMEM as described above containing 10 μmol/liter cytosine arabinoside. 10 μmol/liter cytosine arabinoside was applied for the first 3 days. After overnight incubation, FBS was reduced to 1% (v/v) followed by DMEM lacking serum for at least 24 h. After NRVMs were plated for 6–8 h, adenovirus expressing PLCε siRNA 5, random siRNA, PLCε, PLCε(H1460L), PLCε-RA1, mAKAP-SR1, or YFP as control was added (multiplicity of infection 50) for 4–6 h followed by washing. NRVMs were then cultured in serum-free DMEM medium containing 0.1% (v/v) insulin-transferrin-selenium. After adenovirus infection for at least 2 days and serum starvation for at least 24 h, 10 mmol/liter ET-1, 10 μmol/liter Iso, 10 μmol/liter norepinephrine (NE), or 10 nmol/liter IGF-1 was added for 48 h to induce hypertrophy.

Hypertrophic agonist as described above. Cells were washed twice and solubilized in 0.4N NaOH for 2 h. Total phospholipases (IPs) produced were measured as in Ref. 30.

Measurement of ANF Levels by Real Time PCR—NRVMs were plated in 6-well 0.2% gelatin-coated plates at a density of 5.0 × 10^4 cells/ml and 500 μl/well. For real time PCR, total RNA was extracted from NRVM using the RNeasy mini kit (Qiagen). 300 ng was reverse-transcribed with reverse primers to GAPDH and atrial natriuretic factor (ANF) using SuperScript III reverse transcriptase (Invitrogen). Real time PCR was performed on this product on the BioRadCFX96 real time system with a C 1000 thermal cycler using the iTQ SYBR Green supermix (Bio-Rad).

The primers used were: ANF, 169-bp PCR product, ANF forward (169), 5’-ATCTGATGGATTCAAGAAC-3’; ANF reverse (338), 5’-CTCTGAGAGGGTTGACTTC-3’; GAPDH, 260-bp PCR product, GAPDH forward (648), 5’-AAGGTCTC- CCAGAGCTGAC-3’; GAPDH reverse (908), 5’-TCAATT- GAGAGCAATGCCC-3’. ANF values were normalized to GAPDH, and data collected were analyzed using the GraphPad Prism software. Each experiment was performed 2–3 times independently with results from each experiment giving one data point.

Isolation of Nuclei from NRVMs—The protocol was as previously reported except that the fraction containing SR, plasma membrane, and Golgi bodies was not further purified (31).

Statistical Analysis—For the majority of the [3H]leucine incorporation, cell area, real time PCR, and inositol phosphate measurements, significance was calculated by dividing the mean ligand-stimulated response by the unstimulated response for either random or PLCε siRNA treatment to determine fold activation. The difference in fold activation for random siRNA versus PLCε siRNA was analyzed for statistical significance using a one-tailed unpaired Student’s t test. Selected data in Fig. 3A were analyzed by one-way analysis of variance with Bonferroni’s post test.

RESULTS

PLCε Knockdown in NRVMs Inhibits Endothelin-1-dependent Hypertrophy—We previously established an siRNA protocol for PLCε-specific knockdown in Rat-1 fibroblasts. This siRNA treatment did not affect expression of other PLC isoforms or expression of G proteins, and several siRNA sequences produced similar biological effects (28). We selected the most effective siRNA sequence from those tested (siRNA PLCε 5) that knocked down PLCε by 97% in Rat-1 fibroblasts and created an adenovirus for infection of NRVMs. Supplemental Fig. 1A shows RT-PCR analysis of PLCε RNA extracted from NRVMs infected for 3 and 6 days. PLCε mRNA was effectively depleted after 3 days and remained low after 6 days of viral infection. PLCε protein was also depleted after 3 days of viral infection (supplemental Fig. 1B), whereas the levels of other PLC isoforms were unaffected by the siRNA treatment (supplemental Fig. 1C).

First, we examined the role of PLCε in ET-1-dependent cardiac hypertrophy. ET-1 treatment for 48 h caused a significant increase in three markers of hypertrophy: [3H]leucine incorporation (Fig. 1A), cell area (Fig. 1, B and C), and ANF mRNA (Fig. 1D) in random siRNA infected cells, indicating stimulation of
the hypertrophic response. Surprisingly, PLCε siRNA blocked ET-1-dependent increases in all hypertrophic markers (Fig. 1, A–D), indicating a requirement for PLCε for ET-1-dependent hypertrophy in NRVMs. To show that this result was not due to a nonspecific effect of the PLCε siRNA, we co-expressed PLCε with either a LacZ control or PLCε with a non-coding mutation that provides resistance to the siRNA (PLCε(res)) to replace the endogenously depleted PLCε protein with exogenously expressed PLCε. Leucine incorporation was monitored as a measure of hypertrophy (supplemental Fig. 1D). As in Fig. 1A, PLCε siRNA suppressed ET-1-induced [3H]leucine incorporation, but co-expression with PLCε (Res) prevented this effect, demonstrating rescue of the hypertrophic response upon re-expression of PLCε in the PLCε siRNA background.

Overall, suppression of the hypertrophic response to ET-1 by PLCε depletion was unexpected because it is the opposite of what was observed in the PLCε−/− mice where the loss of PLCε appeared to increase hypertrophy. Regardless of this apparent contradiction, this result was also unexpected because ET-1 is thought to stimulate phosphatidyl inositol hydrolysis through a Gq-dependent mechanism that would not be expected to directly involve PLCε. ET4 receptors can also signal via G12/13, Rho-1, G1/βγ, and G0-dependent pathways, and it is possible that PLCε is regulated through one of these signaling mechanisms. It was noted that PLCε knockdown alone increased cell area in the absence of stimulation by ET-1, but this appears to be unrelated to the hypertropic response because the other markers of hypertrophy, ANF mRNA levels and protein synthesis, are not significantly altered by PLCε siRNA treatment in the absence of stimulation by agonist.

PLCε Is a Central Participant in Ligand-dependent Hypertrophic Responses in NRVMs—PLCε has the potential to be regulated by multiple upstream signaling mechanisms. We have extensively characterized a role in cAMP-Dependent Rap-dependent signaling, and PLCε can respond to multiple G protein-coupled receptor-dependent and receptor tyrosine kinase-dependent pathways through Ras, Rho, Gi/βγ, or other potential regulatory molecules (13, 16, 17, 32). For this reason, we examined the effects of PLCε knockdown on stimulation of hypertrophy by agonists that signal through distinct cellular mechanisms. Iso likely works through a cAMP-dependent mechanism. NE activates Gs-dependent signaling via stimulation of α1-adrenergic receptors in neonatal myocytes (33). IGF-1 signals via tyrosine phosphorylation and couples to PLCγ (8). Again, PLCε knockdown almost completely inhibited ligand-dependent hypertrophy by all of these agonists (Fig. 2 and supplemental Fig. 2), indicating a central role of PLCε in hypertrophic signaling by diverse hormonal regulatory mechanisms.

To determine whether PLCε overexpression can promote hypertrophy in NRVMs, cells were infected with an adenovirus expressing PLCε under the control of a mouse cytomegalovirus promoter, and [3H]leucine incorporation was measured. PLCε expression alone significantly increased leucine incorporation (Fig. 3A), indicating that increasing PLCε expression was sufficient to increase hypertrophy. ET-1 was able to further increase

**FIGURE 1.** PLCε is required for ET-1-dependent cardiomyocyte hypertrophy. NRVMs were infected with PLCε-siRNA or random siRNA (Ran siRNA) adenoviruses followed by stimulation with 10 nmol/liter ET-1 as described under “Experimental Procedures.” A, [3H]leucine incorporation was measured as described under “Experimental Procedures.” Data are combined results from four separate preparations of myocytes with each experiment performed in triplicate. Con, control. B, NRVMs were fixed and stained with a-actinin antiserum as described under “Experimental Procedures.” Scale bar is 100 μm. C, NRVM surface areas were calculated using the NIH ImageJ software from over 400 myocytes for each condition. Data were pooled from 2 independent experiments. D, ANF mRNA was measured by real time PCR and normalized to GAPDH as described under “Experimental Procedures.” Data are combined from two different preparations of cells. Error bars indicate S.E. *, p < 0.05, ***, p < 0.001.
[3H]leucine incorporation in the context of PLCε overexpression. To determine whether PLCε catalytic activity was required for the PLCε-dependent increase in hypertrophy, we expressed PLCε with a point mutation in the catalytic domain that eliminates PLC hydrolytic activity (PLCε(H1460L)) and measured hypertrophy. Expression of PLCε(H1460L) did not stimulate hypertrophy when compared with the YFP control. Expression of PLCε(H1460L) also suppressed hypertrophy stimulated by ET-1, likely acting in a dominant negative manner (Fig. 3A). Adenoviral expression of PLCε and PLCε(H1460L) gives equal levels of PLCε protein in ventricular myocytes (not shown). These data indicate that the products of PLCε catalytic activity are important for the hypertrophic response. To examine the role of PLCε in receptor-driven phosphatidylinositol 4,5-bisphosphate hydrolysis, we knocked down PLCε and PLCε(H1460L) with siRNA and examined ET-1- and NE-stimulated IP production. PLCε knockdown had no effect on the ability of these agonists to stimulate total IP production (Fig. 3, B and C). These data indicate that the products of PLCε activity are important for the hypertrophic response. To examine whether PLCε catalytic activity was required for the PLCε-dependent increase in hypertrophy, we expressed PLCε with a point mutation in the catalytic domain that eliminates PLC hydrolytic activity (PLCε(H1460L)) and measured hypertrophy. Expression of PLCε(H1460L) did not stimulate hypertrophy when compared with the YFP control. Expression of PLCε(H1460L) also suppressed hypertrophy stimulated by ET-1, likely acting in a dominant negative manner (Fig. 3A). Adenoviral expression of PLCε and PLCε(H1460L) gives equal levels of PLCε protein in ventricular myocytes (not shown). These data indicate that the products of PLCε catalytic activity are important for the hypertrophic response. To examine the role of PLCε in receptor-driven phosphatidylinositol 4,5-bisphosphate hydrolysis, we knocked down PLCε and PLCε(H1460L) with siRNA and examined ET-1- and NE-stimulated IP production. PLCε knockdown had no effect on the ability of these agonists to stimulate total IP production (Fig. 3, B and C). We propose that PLCε activity does not significantly contribute to global cellular IP production either because it is expressed at very low levels, because its localization is restricted, or both. Possible mechanisms for subcellular localization of activity are discussed in depth below. Overall, these data suggest that PLCε is necessary for hypertrophy driven by multiple neurohumoral stimuli and that PLCε activity is sufficient to drive hypertrophic responses.

**mAKAP Binds PLCε in Transfected Cells and in the Heart—**
Because the data suggest that PLCε may be acting locally to produce its physiological effects and because one of the regulators of PLCε, Epac, scaffolds to mAKAPβ, we tested whether PLCε could interact with mAKAPβ. Myc-mAKAP was co-expressed with PLCε in HEK293 cells followed by immunoprecipitation of mAKAP and immunoblotting for either PLCε or mAKAP (Fig. 4A). PLCε was found in Myc-mAKAP immunoprecipitates only when both proteins were expressed. No PLCε immunoreactivity was detected if either PLCε or Myc-mAKAP was absent. To determine whether mAKAPβ and PLCε associate in cardiac tissue, mouse heart lysates were prepared from PLCε+/+ and PLCε−/− mice followed by PLCε immunopre-
was mutated to proline in the mAKAP spectrin repeat-like domain 1, and binding of this mutant protein fused to GST to purified PLCε was examined (Fig. 5D). This mutation strongly decreased binding of PLCε to this domain, confirming that PLCε binds to the first spectrin repeat-like domain in mAKAP.

Identification of PLCε Functional Domains Required for mAKAP Interactions—To identify specific domains in PLCε that mediate mAKAP binding, PLCε truncated at the C terminus (ΔCT) to delete the Ras association (RA) domains or PLCε with a GEF domain deletion (ΔGEF) was co-expressed with Myc-mAKAP in HEK293 cells (Fig. 6A). The ΔCT mutant was unable to interact with mAKAP in co-immunoprecipitation assays, whereas the interaction was unaffected by the ΔGEF mutation (Fig. 6B). Next, either the PLCε-RA1 or the PLCε-RA2 domains were expressed with Myc-mAKAP followed by Myc-mAKAP immunoprecipitation. Both PLCε-RA1 and PLCε-RA2 independently bound to co-expressed mAKAP (Fig. 6C). These data indicate that the RA domains are necessary and sufficient for binding of PLCε to mAKAP. Given that the RA domains are important for Rap and Ras interactions, we determined whether activated RapG12V or RasG12V altered PLCε-mAKAP binding in transfected cells. Expression of activated Rap or Ras did not affect PLCε-mAKAP co-immunoprecipitation (data not shown).

Disruption of mAKAP-PLCε Binding by Expression of PLCε RA1 or mAKAP-SR1—To determine whether expression of PLCε-RA1 or mAKAP-SR1 could compete for PLCε binding to mAKAP, and PLCε and mAKAP were co-expressed in HEK293 cells, plus or minus co-expression of PLCε-RA1 or mAKAP-SR1 domains. Myc-mAKAP protein complexes were immunoprecipitated with Myc antibodies and immunoblotted for PLCε. PLCε co-immunoprecipitated with mAKAP, but expression of either PLCε-RA1 or mAKAP-SR1 prevented mAKAP-PLCε co-immunoprecipitation (Fig. 7A). To test whether PLCε-RA1 or mAKAP-SR1 can disrupt endogenous mAKAP-PLCε signaling complexes in NRVMs, NRVMs were infected with adenoviruses expressing either domain followed by immunoprecipitation of mAKAP and immunoblots of PLCε. PLCε co-immunoprecipitated with mAKAP when YFP was expressed as a control (Fig. 7B), although the complex was difficult to detect. In general, endogenous PLCε is difficult to detect and has to be enriched by direct IP from most tissues to enable adequate detection by immunoblotting. Nevertheless, expression of either PLCε-RA1 or mAKAP-SR1 in NRVMs significantly reduced the association of PLCε with mAKAP immunoprecipitates (Fig. 7B), indicating that expression of either of these domains disrupts endogenous mAKAPβ-PLCε complexes in NRVMs.

To confirm the location of endogenous mAKAPβ in NRVMs, isolated myocytes were fixed and stained with mAKAP antibody (data not shown). As reported previously, there was a very strong perinuclear staining of mAKAP (25). Reliable detection of PLCε by immunocytochemistry was not possible, likely due to its low abundance coupled with nonspecific background staining with PLCε antibodies. Given the interaction with mAKAPβ and the predominant perinuclear localization of mAKAP, it is likely that PLCε interaction with
mAKAPβ localizes some of the PLCε pool to the perinuclear region of the cell. To confirm the nuclear localization of PLCε, NRVMs were fractionated to isolate nuclei and immunoblotted for PLCε. PLCε was significantly enriched in the nuclear fraction when compared with whole cell NRVM lysate, consistent with association with nuclear-bound mAKAP. Nuclear enrichment was confirmed by measuring the enrichment of the nuclear protein LAP-2 and mAKAP. Both proteins were greatly enriched relative to the whole cell NRVM lysates. To confirm that the nuclei were free of SR, the lysates were probed for calsequestrin, which was enriched in an SR fraction but was absent from the nuclear fraction.

mAKAPβ spectrin repeat domain 3, but not repeats 1 or 2, has been shown to mediate interactions with nesprin-1α, which localizes mAKAP to the perinuclear region (25) of cardiac myocytes. Nevertheless, because PLCε interacts with spectrin repeat domain 1, we tested whether expression of either PLCε-RA1 or mAKAP-SR1 disrupted perinuclear localization of mAKAPβ in NRVMs. Neither domain altered the localization of mAKAPβ as assessed by immunocytochemistry of NRVMs.
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**FIGURE 7.** The PLC-RA1 and mAKAP-SR1 domains compete for mAKAP-PLCε interactions. **A**, PLCε and Myc-mAKAP were cotransfected (Transfect.) into HEK393 cells in the presence or absence of PLCε-RA1 or mAKAP-SR1 domains. Myc-mAKAP was immunoprecipitated (IP) and Western blotted (IB) for PLCε (top panel) or Myc (bottom panel). Each experiment was repeated at least 3 times with similar results. **B**, NRVMs were infected with adenoviruses expressing YFP, PLCε-RA1, or mAKAP-SR1 for 48 h. Lysates were prepared (1 mg of protein, PLCε-RA1-infected cells, or 2 mg of protein, mAKAP-SR1-infected cells), and mAKAPβ was immunoprecipitated. Immunoprecipitates were immunoblotted for either PLCε or mAKAPβ. This experiment was repeated twice. **C**, NRVMs were fractionated to isolate nuclei and 10 μg of protein was analyzed by Western blotting. SR/PM, sarcoplasmic reticulum/plasma membrane.

(supplemental Fig. 3), indicating that these domains effectively disrupt endogenous mAKAPβ-PLCε interactions without influencing mAKAPβ localization to the nuclear envelope. Thus, expression of PLCε-RA1 or mAKAP-SR1 likely disrupts PLCε localization at the nuclear envelope, while leaving the mAKAPβ scaffold itself undisturbed.

**Importance of PLCε Scaffolding to mAKAP in Cardiomyocyte Function**—We have shown that PLCε is an important central mediator of hypertrophy and that it is scaffolded to mAKAPβ, which we have also shown to be important for cardiac myocyte hypertrophy (16). To establish the functional relevance of mAKAPβ-PLCε complexes, we tested whether mAKAPβ-PLCε binding was required for the hypertrophy of NRVMs. NRVMs were infected with an adenovirus expressing either PLCε-RA1 or mAKAP-SR1 to disrupt PLCε-mAKAPβ binding followed by treatment with 10 nm ET-1 for 48 h. Expression of either PLCε-RA1 or mAKAP-SR1 significantly blunted ET-1-dependent increases in [3H]leucine incorporation and ANF mRNA expression (Fig. 8, A and B). PLCε-RA1 is related to Ras binding domains but does not bind Ras or Rap (34). Nevertheless, to be sure that PLCε-RA1 was not inhibiting hypertrophy by blocking Ras or Rap signaling, we measured IGF-1-stimulated ERK phosphorylation, in the presence or absence of PLCε-RA1 expression. The IGF-1-stimulated increase in ERK1/2 phosphorylation in NRVMs was not affected by expression of PLCε-RA1 (data not shown), indicating that these proteins do not globally interfere with Ras signaling. That two independent approaches to disrupting mAKAPβ-PLCε complexes, using two different binding domains from two different proteins, both significantly inhibited ET-1-dependent hypertrophy strongly suggests that scaffolding of PLCε to mAKAPβ is required for hypertrophic responses mediated by ET-1. PLCε-RA1 expression in NRVMs also significantly inhibited [3H]leucine incorporation in response to Iso, NE, and IGF-1, indicating a general role for nuclear scaffolding of PLCε (supplemental Fig. 4).

**DISCUSSION**

Here we demonstrate that PLCε plays a central role in mediating hypertrophy downstream of multiple agonists and that subcellular scaffolding of PLCε to mAKAPβ, likely at the nuclear envelope, is critical for this process. Mechanistically, the upstream pathways that regulate PLCε for these hypertrophic responses remain to be defined in NRVMs. Possible mechanisms for activation include G_{12/13}-dependent Rho pathways, cAMP-Epac pathways, and Ras pathways. ETA receptors can activate G_{12/13}, IGF-1 receptors can activate Ras, and βAR can activate cAMP-Epac-Rap, all of which are potential PLCε regulators in NRVMs. α1-Adrenergic receptors signal primarily through G_{q}, which would not activate PLCε, but G_{q} activation can lead to activation of Rho and Ras pathways that could regulate PLCε activity. That PLCε can respond to such diverse signals ideally positions it to be a central integrator of multiple hormonal signals that impinge on the cardiac myocytes during physiologic and pathologic stimulation.
Regardless of the mechanism for activation, the catalytic activity of PLCε is required for hypertrophy because expression of a catalytically inactive form of PLCε does not stimulate hypertrophy and blocks development of ET-1-dependent hypertrophy. PLC activity results in the production of two second messengers, DAG and IP₃, and roles for both of these molecules have been suggested in hypertrophy. Nevertheless, the data show that PLCε is not responsible for the majority of global IP₃ generation downstream of ETₐ or α1AR. An attractive hypothesis to explain this is that PLCε scaffolded to mAKAPβ at the nuclear envelope generates IP₃, and DAG locally to regulate nuclear Ca²⁺ or PKC signals without significantly contributing to the pool of total IPs. In ventricular myocytes, IP₃ receptors are localized primarily to the nuclear envelope and release a pool of nuclear calcium distinct from that involved in calcium-induced calcium release (35, 36). This nuclear calcium pool has been implicated in regulating hypertrophic gene expression through two pathways: (i) activation of nuclear CamKII leading to phosphorylation of histone deacetylase 5 (HDAC5) and derepression of myocyte enhancer factor (MEF)-dependent transcription; and (ii) activation of calcineurin-dependent dephosphorylation of nuclear factor of activated T-cells (NFAT) leading to increased nuclear factor of activated T-cell-dependent transcription. Additionally, PKC activation has been implicated in hypertrophy, and PKC-PKD-dependent phosphorylation of HDAC leads to export of HDAC5 from the nucleus (37). PLCε could be involved in the local generation of either IP₃ or DAG at the nuclear envelope necessary for these processes.

The PLC isoform responsible for the majority of IP₃ production downstream of Gαq-coupled receptors has been suggested to be PLCβ because the major known target of Gαq is PLCβ (6, 38), although Gαq can also regulate Rho activation through direct stimulation of RhoGEFs (39). Data directly implicating PLCβ in cardiac hypertrophy have been lacking until recently. These new data show that PLCβ1b overexpression increases NRVM hypertrophy and that inhibition of PLCβ1b sarcolemmal membrane association can inhibit α1AR-dependent hypertrophy (9). Our published results in astrocytes (17) and fibroblasts (28) indicate that surprisingly, many Gαq-coupled receptors generate significant accumulation of total IPs through PLCε, suggesting the possibility that some portion of hypertrophy driven through Gαq-linked receptors could be downstream of PLCε activation. Nevertheless, the bulk of IP₃ generation in NRVMs does not appear to involve PLCε and is likely PLCβ-driven, suggesting that PLCε-dependent local phosphatidylinositol 4,5-bisphosphate hydrolysis, distinct from the bulk IP₃ pool, is required for hypertrophic responses. It is possible that hypertrophy in response to ET-1 and NE is both PLCβ1b-dependent and PLCε-dependent and that two distinct pools of IP₃ and/or DAG are required for the hypertrophic response.

We previously reported that PLCε activity is important for CamKII-dependent phosphorylation of Ryr2 and regulation of cardiac Ca²⁺ cycling (41). Based on this observation, we predict that mAKAPβ scaffolded PLCε represents a pool of PLCε that is distinct from a separately scaffolded pool that regulates RyR2. mAKAPβ has been reported to bind to RyR2 (26), but this is at odds with the observation that the majority of mAKAPβ is found at the nuclear envelope, whereas the bulk of RyR2 protein is in the SR. Nevertheless, it remains possible that a small pool of mAKAPβ can scaffold PLCε to RyR2. This hypothesis remains to be investigated.

Our data demonstrating a requirement for PLCε in hormone-regulated hypertrophic responses contrast with our previously reported in vivo observation that global PLCε⁻/⁻ mice are more sensitive to the development of hypertrophy in response to chronic Iso treatment (18). A possible explanation for the difference in the cellular and in vivo data is that in the context of development of the PLCε⁻/⁻ mice, compensatory pathways were up-regulated that sensitized mice to hypertrophy. In particular, we found that CamKII phosphorylation is basally increased in PLCε⁻/⁻ mice (supplemental Fig. 5), which could lead to enhanced sensitivity to stress-induced hypertrophy because CamKII activation has been shown to be prohypertrophic (40). CamKII activation is not elevated in PLCε siRNA-treated myocytes (data not shown). It is not unusual for compensatory pathways to be up- or down-regulated in global knock-out mice where the gene product is lost from birth. This suggests that our previous conclusion from global PLCε⁻/⁻ mice suggesting a protective role for PLCε was incorrect. Analysis of mice with cardiac myocyte-specific, acute PLCε knock-down would help to resolve this discrepancy.

Overall, these data demonstrate that PLCε plays a central role in mediating hypertrophy in response to multiple neurohumoral stimuli and that scaffolding to mAKAPβ is critical for this. The human heart is exposed to multiple hormonal inputs during the development of heart failure. PLCε represents a potentially interesting therapeutic target as a central integrator of these inputs, possibly allowing for more efficacious treatment of heart failure. Because PLCε plays an important role in the CNS, pancreas, and kidney as well as other tissues, targeting PLCε activity directly might not be desirable. Development of an approach that could specifically interfere with PLCε-mAKAPβ interactions could provide a more selective approach to targeting PLCε and its role in hypertrophy.

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