The Phosphatidylinositol(4,5)Bisphosphate–Binding Sequence of Transient Receptor Potential Channel Canonical 4α Is Critical for Its Contribution to Cardiomyocyte Hypertrophy

Nicola Cooley, David R. Grubb, Jieting Luo, and Elizabeth A. Woodcock

Molecular Cardiology Laboratory, Baker IDI Heart and Diabetes Institute, Melbourne, Victoria, Australia

Received May 12, 2014; accepted July 21, 2014

ABSTRACT

Cardiomyocyte hypertrophy requires a source of Ca2+ distinct from the Ca2+ that regulates contraction. The canonical transient receptor potential channel (TrpC) family, a family of cation channels regulated by activation of phospholipase C (PLC), has been implicated in this response. Cardiomyocyte hypertrophy downstream of Gq-coupled receptors is mediated specifically by PLCb1 that is scaffolded onto a SH3 and ankyrin repeat protein 3 (Shank3) complex at the sarcolemma. TrpC4 exists as two splice variants (TrpC4α and TrpC4β) that differ only in an 84-residue sequence that binds to phosphatidylinositol(4,5) bisphosphate (PIP2), the substrate of PLCb1. In neonatal rat cardiomyocytes, TrpC4α, but not TrpC4β, comimmunoprecipitated with both PLCb1 and Shank3. Heightened PLCb1 expression caused TrpC4α, but not TrpC4β, translocation to the sarcolemma, where it colocalized with PLCb1b. When overexpressed in cardiomyocytes, TrpC4α, but not TrpC4β, increased cell area (893 ± 18 to 1497 ± 29 mm², P < 0.01) and marker gene expression (atrial natriuretic peptide increased by 409 ± 32%, and modulatory calcineurin inhibitory protein 1 by 315 ± 28%, P < 0.01). Dominant-negative TrpC4 reduced hypertrophy initiated by PLCb1b, or PLCb1b-coupled receptor activation, by 72 ± 8% and 39 ± 5%, respectively. We conclude that TrpC4α is selectively involved in mechanisms downstream of PLCb1b culminating in cardiomyocyte hypertrophy, and that the hypertrophic response is dependent on the TrpC4α splice variant-specific sequence that binds to PIP2.

Introduction

Hypertrophic growth of the myocardium is a complex response initiated by pathologic stimuli, including pressure or volume overload, myocardial infarction, and congenital factors. Significantly, pathologic hypertrophic responses require the participation of G proteins of the Gq family (Akhter et al., 1998; Dorn and Brown, 1999). Despite this, the immediate signaling responses to Gq activation in heart, and their relationships to cellular growth, remain poorly understood. In cardiomyocytes, Gq activates one splice variant of phospholipase C (PLC) b1, PLCb1b, exclusively (Grubb et al., 2008; Fitzl et al., 2009), and PLCb1b is the only PLC that induces hypertrophy when overexpressed in cardiomyocytes in vitro. PLCb1b differs from the other splice variant PLCb1a by only a 32-residue C-terminal sequence, and the splice variant specificity allowed us to identify factors critically involved in Gq/PLCb1b-mediated responses. Important among these are the scaffolding proteins, SH3 and ankyrin repeat protein 3 (Shank3) and Homer1c (Grubb et al., 2011; Grubb et al., 2012), both of which form complexes specifically incorporating PLCb1b. Within central neurons, Shank3 and Homers are associated with transient receptor potential channels (TrpC) (Kaznacheyeva et al., 2007; Bertaso et al., 2010). This is of interest because TrpC are implicated in Ca2+ responses specifically targeted to hypertrophic responses (Wu et al., 2010; Eder and Molkentin, 2011; Gomez et al., 2013).

TrpC are nonselective, low-conductance channels that mediate entry of calcium and sodium, in response to activation of receptors coupled to PLC. The association between TrpC and PLC activation is complex and differs between the TrpC subtypes, as well as the cell type of origin (Hardie, 2003). PLC activation generates inositol(1,4,5)trisphosphate (IP3) and sn-1,2-diacylglycerol (DAG) at the expense of the substrate lipid, phosphatidylinositol(4,5)bisphosphate (PIP2), and each of these, IP3, DAG, and PIP2, can influence TrpC activity independently as well as in concert. TrpC form homotetramers as well as heterotetramers with other TrpC, on a subtype-selective basis. TrpC1, -4, and -5 constitute one subfamily, and TrpC3, -6, and -7 another. TrpC3, -6, and -7 are activated directly by DAG as well as indirectly following DAG-induced protein kinase C activation (Hardie, 2003). In contrast, TrpC1, -4, and -5 do not respond to DAG, although TrpC1 can be activated by protein kinase C, and thus indirectly by DAG.

This work was supported by the Australian National Health and Medical Research Council [Grants 1002328, 10007712, and 1022678]; the Victorian Government’s Operational Infrastructure Support Program; and a Baker IDI Heart and Diabetes Institute commercialization grant. E.A.W. is a fellow of the Australian National Health and Medical Research Council [Grant 586621]. dx.doi.org/10.1124/mol.114.093690.

ABBREVIATIONS: ANP, atrial natriuretic peptide; DAG, sn-1,2-diacylglycerol; FBS, fetal bovine serum; MCIP, modulatory calcineurin inhibitory protein 1; NRV, neonatal rat ventricular myocyte; PBS, phosphate-buffered saline; PDZ, post synaptic density protein (PSD95); Drosophila large tumor suppressor, and zona occulens-1 protein (zo-1); PIP2, phosphatidylinositol(4,5)bisphosphate; PLC, phospholipase C; Shank3, SH3 and ankyrin repeat protein 3; TrpC, transient receptor potential channel; TTRL, threonine, threonine, arginine, leucine.
Cardiomyocytes express multiple subtypes of TrpC, including TrpC1, -3, -4, -5, and -6 (Watanabe et al., 2009; Eder and Molkentin, 2011), making evaluation of individual contributions difficult, especially when the functional channels can be heteromeric. TrpC3 and TrpC6 are elevated in hypertrophy and heart failure, and both have been suggested to supply the Ca^{2+} to activate responses downstream of calcineurin (Onohara et al., 2006). In addition to the DAG-regulated TrpC3/6 channels, there has also been evidence for an involvement of the DAG-insensitive TrpC family members, TrpC1 and TrpC4. TrpC1 expression is heightened in hypertrophy models (Kiso et al., 2013) and contributes to pressure overload hypertrophy (Seth et al., 2009). Dominant-negative TrpC4 reduces hypertrophy following pressure overload or infusion of the Gq/PLC-coupled agonist, angiotensin II (Kiselyov et al., 2005) suitable for binding directly to the PDZ domain of Shank3. TrpC4 has previously been shown to colocalize with PLC subtypes based on associations with PDZ-containing scaffolding proteins via the TTRL sequence (Tang et al., 2000). Shank3 may fulfill a scaffolding role in organizing signaling complexes incorporating PLCβ1 and TrpC4/5 in cardiomyocytes (Grubb et al., 2011).

The initiation of hypertrophic signaling downstream of Gq in cardiomyocytes depends on a protein complex incorporating Shank3 and PLCβ1b, and we considered the possibility that TrpC family members also associated with this complex. The DAG-insensitive TrpC family members, TrpC4 and TrpC5, have an identical C-terminal PDZ-interacting motif (TTRL) (Kiselyov et al., 2005) suitable for binding directly to the PDZ domain of Shank3. TrpC4 has previously been shown to colocalize with PLC subtypes based on associations with PDZ-containing scaffolding proteins via the TTRL sequence (Tang et al., 2000). Shank3 may fulfill a scaffolding role in organizing signaling complexes incorporating PLCβ1b and TrpC4/5 in cardiomyocytes (Grubb et al., 2011).

In the current study, we investigated whether TrpC4/5 associated with the Shank3/PLCβ1b complex in cardiomyocytes and whether any PLCβ1b/Shank3-associated TrpC contributed to hypertrophic responses. We found that only one of the splice variants of TrpC4, specifically TrpC4α, which incorporates a PIP2-binding domain, associated with the complex. Furthermore, expression of TrpC4α, but not TrpC4β, was sufficient to cause cardiomyocyte hypertrophy, even though these two proteins differ by only an 84-amino-acid, PIP2-binding sequence near to the C terminus of the proteins.

Materials and Methods
Preparation of Neonatal Rat Ventricular Myocytes. Experiments were approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee, and all work was performed in accordance with the Australian National Health and Medical Research Council provision for the care and use of laboratory animals. Ventricular myocytes were prepared from 1- to 2-day-old Sprague-Dawley rats of either gender, using repeated pancreatin/collagenase digestion, followed by separation from nonmyocytes using discontinuous Percoll gradients, as described previously (Sah et al., 1996). Cells were plated at 400/mm² and were maintained in defined medium comprising Dulbecco’s modified Eagle’s medium, insulin (50 μg/ml), transferrin (10 μg/ml), sodium selenate (30 nM), bromoexoyuridine (0.1 mmol/l), and antibiotics. Cells were used within 3 days of preparation. Bromoexoyuridine was omitted after 3 days.

Measurement of Neonatal Rat Ventricular Myocyte Hypertrophy. Cell area was quantified from manually-outlined cells in digitized microscopic images (recorded by an Olympus phase-contrast microscope; Olympus, Melbourne, Australia) of randomly chosen cell fields using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD). A minimum of 40 cells over four wells per treatment was measured for each independent experiment, and the experiments were repeated at least three times. Protein was measured using the bicinchoninic acid method for protein (Thermo Scientific, Melbourne, Australia) and Burtons’ diphenylamine procedure for measurement of DNA, as described previously (Filtz et al., 2009).

Constructs and Adenoviruses. Adenoviruses expressing FLAG-PLCβ1b and PLCβ1a have been described previously (Filtz et al., 2009). cDNAs for TrpC4α and TrpC4β were provided by J. Putney (Research Triangle Park, NC) (McKay et al., 2000). cDNA for dn-TrpC4 N-terminally myc-tagged (Schindl et al., 2006; Wu et al., 2010) was provided by DNA 2.0 (Menlo Park, CA). Adenoviruses expressing TrpC4 were generated using the Gateway methodology (Life Technologies, Melbourne, Australia) and were used at 20–50 plaque-forming units per cell.

Commmunoprecipitation and Western Blotting. Neonatal rat ventricular myocytes (NRVM) plated on 9-cm dishes were washed with Hanks’ buffered salt solution and then lyzed and harvested in buffer containing the following, in mmol/l: 50 HEPES (pH 7.4), 130 NaCl, 2 MgCl2, 1 CaCl2, 40 KKH2PO4, as well as 1% (v/v) Triton X-100, 0.5% Nonidet P40, 15% glycerol, 0.2% (w/v) bovine serum albumin, plus a protease inhibitor cocktail (Roche, Sydney, Australia). The extract was precleared, and then anti-FLAG antibody (FLAG-M2; Sigma-Aldrich, St. Louis, MO), anti-TrpC4 (Millipore), anti-Shank3 (Millipore), or anti-myc (Abcam) antibody was added at 5 μl/ml extract, followed by protein A or protein G Sepharose at 50 μl/ml extract. After overnight incubation at 4°C, antibody-bound material was harvested by centrifugation, and the pellets were washed three times with lysis buffer containing protease inhibitors. Proteins were separated by SDS-PAGE using gradient 7.5–15% gels and were transferred to nitrocellulose membranes. Antibodies were used at the following dilutions: Shank3 1/2000, FLAG 1/1000, myc 1/5000, TrpC4 1/500, and TrpC5 1/500 (Alomone). All blots were developed using horseradish peroxidase–conjugated secondary antibodies, and images were quantified using a GelDoc XR+ system using ImageLab 2.0.1 software (Bio-Rad, Sydney, Australia).

Membrane Raft Separation. Membrane fractions were separated on the basis of buoyant density using detergent-free methodology, as described previously (Morris et al., 2006). The 2:1 ml fractions were collected, diluted, and pelleted at 75,000g prior to Western blotting. Bands corresponding to TrpC4α and TrpC4β in the pelleted fractions were quantified relative to bands in the original lysate (prior to gradient centrifugation).

Confocal Microscopy. NRVM were plated onto gelatinized glass-bottom confocal dishes (MatTek, Ashland, MA) and infected with adenovirus, as indicated. Treated NRVM were fixed with paraformaldehyde (4% w/v, 0.1 M PIPES, pH 6.8) before permeabilization with saponin buffer (0.05% w/v, 0.1 M PIPES, pH 6.8). The plates were washed in phosphate-buffered saline (PBS) and incubated with antibodies diluted in PBS plus bovine serum albumin 1% w/v. Plates were then further washed in PBS and incubated with AlexaFluor-488– or AlexaFluor-546–conjugated secondary antibodies (Life Technologies). The samples were then further washed in PBS and mounted using Vectashield mounting media (Abacus ALS, Brisbane, Australia). Images were captured using a Zeiss Meta-510 LSM (excitation 488 nm and 543 nm, with emission at 519 nm and 573 nm).

Measurement of mRNA Expression. RNA was extracted using RNEasy kits from Qiagen (Melbourne, Australia), according to the manufacturer’s instructions, and reverse transcribed using Superscript III (Life Technologies). Real-time quantitative reverse-transcription polymerase chain reaction with SybrGreen (Life Technologies) reagent was performed on an Applied Biosystems 7500 Fast Real Time PCR System using primers for rat glyceraldehyde-3-phosphate dehydrogenase (5′-CAATATGATTCTACCCACGG-3′ and 5′-CAGATCCACAAAGGAGTACAT-3′), which were used as a reference gene, and atrial natriuretic peptide (ANP) (5′-AGCCTGCGAAGGTCAAGCT-3′ and 5′-CGTCCCAATCTGTCAGGAC-3′) modulatory calceinin inhibitor protein 1 (MCIP) (5′-TGCGGGCGAAGAGGTAAGATG-3′ and 5′-TGACTGGGTAGCGTCTTC-3′). Values are expressed as the 2−ΔCt value relative to glyceraldehyde-3-phosphate dehydrogenase in each sample (Pfaffl, 2001).
chain reaction experiments were performed in triplicate on triplicate or quadruplicate samples.

**Statistics.** Values are shown as mean ± S.E.M. Comparisons between treatment groups were carried out using one-way analysis of variance, followed by Fisher’s exact test (Sigma Stat). Paired studies were evaluated using a paired t test.

**Results**

**TrpC4α Associates with PLCβ1b and Shank3.** We have previously reported that PLCβ1b associates with Shank3 in cardiomyocytes (Grubb et al., 2011), and we next examined whether either TrpC4 or TrpC5 also associates with this complex. NRVM were treated with Ad-FLAG-PLCβ1b for 24 h; extracts were prepared and immunoprecipitated with anti-TrpC4 or anti-TrpC5 antibodies; and subsequent Western blots were developed with anti-FLAG antibody (Fig. 1, A and C). Anti-TrpC4 antibody precipitated FLAG-PLCβ1b and Shank3 (Fig. 1, A and B), and, similarly, TrpC4 was precipitated by anti-FLAG or anti-Shank3 antibodies (Fig. 1B). In contrast, anti-TrpC5 antibody did not precipitate either FLAG-PLCβ1b or Shank3 (Fig. 1, C and D), and anti-FLAG or anti-Shank3 antibodies did not precipitate TrpC5 (Fig. 1D).

TrpC4 exists as two different splice variants that differ only by an 84-amino-acid sequence close to the C-terminal end of the protein (Fig. 2A) (Otsuguro et al., 2008). Western blots depicted in Fig. 1, A and B, show that only a single band is associated with PLCβ1b or Shank3, and we next undertook studies to identify which of the two splice variants was bound. N-terminally myc-tagged TrpC4α and TrpC4β were expressed in NRVM, along with FLAG-PLCβ1b. Extracts were prepared and immunoprecipitated with anti-myc or anti-FLAG antibodies. Both myc-TrpC4α and myc-TrpC4β were precipitated with anti-myc antibodies, but only myc-TrpC4α was precipitated by anti-FLAG antibody (Fig. 2B) or by anti-Shank3 antibodies (Fig. 2C). Similarly, anti-myc antibody only precipitated FLAG-PLCβ1b or Shank3 when myc-TrpC4α was expressed (Fig. 2D). Anti-myc antibody did not precipitate either FLAG-PLCβ1b or Shank3 when myc-TrpC4β was expressed. Thus, TrpC4α, but not TrpC4β, associates with the PLCβ1b/Shank3 complex in NRVM.

**PLCβ1b Causes Translocation of TrpC4α to Sarcolemmal Fractions.** In cardiomyocytes, PLCβ1b and its scaffold Shank3 are localized primarily in light lipid raft fractions of the sarcolemma along with the PLC substrate PIP2 (Morris et al., 2006; Grubb et al., 2011). We examined the distribution of TrpC4α and TrpC4β between membrane fractions of different buoyant densities, and whether this distribution was altered by expressing PLCβ1b. When extracts from control NRVM were subjected to sucrose gradient centrifugation to separate membranes of different buoyant densities, TrpC4α was found in both heavy and light membrane fractions, at the bottom (fractions 1 and 2) and the top (fractions 5 and 6) of the gradient, respectively (Fig. 3A). Expression of FLAG-PLCβ1b resulted in an increase in TrpC4α in membrane fractions of low buoyant density, without any change in total TrpC4α expression (Fig. 3, A–C). TrpC4β was not detected in light membrane fractions in the absence or presence of FLAG-PLCβ1b. FLAG-PLCβ1b expression did not alter the distribution between heavy and light fractions of TrpC1, or TrpC5.

Using immunofluorescence and confocal microscopy, we examined the localization of TrpC4 in NRVM and whether PLCβ1b influenced the localization. In control NRVM, TrpC4 was present in intracellular vesicles distributed throughout the cytosol [anti-TRpC4 antibody, AlexaFluor-546 (red)] (Fig. 3D). Images were cross sectioned, as indicated by the white lines in Fig. 3D, and pixel density was quantified for FLAG-PLCβ1b and TrpC4 (Fig. 3E). Increasing the expression of PLCβ1b, by treatment with Ad-FLAG-PLCβ1b for 24 hours, resulted in translocation of TrpC4 to the sarcolemma, where it colocalized with FLAG-PLCβ1b [anti-FLAG antibody, AlexaFluor-488 (green)]. Colocalization of TrpC4 and FLAG-PLCβ1b appears as yellow (Fig. 3D) and as colocalized bands (Fig. 3E).

**TrpC4α, but Not TrpC4β, Is Sufficient to Cause Hypertrophy of NRVM.** As noted above, TrpC4 exists as two splice variants, of which only TrpC4α was shown to be associated with PLCβ1b signaling (Figs. 1–3). We next expressed either TrpC4α or TrpC4β (Ad-myc-TrpC4α or Ad-myc-TrpC4β) in NRVM for 24 hours and examined the effect on hypertrophic markers. TrpC4α and TrpC4β, both myc-tagged, were expressed at similar levels in NRVM following treatment with adenovirus for 24 hours (Fig. 4A). Increasing the expression of TrpC4α promoted cardiomyocyte hypertrophy, indicated by increased cell area and protein/DNA ratio (Fig. 4, B and C), as well as hypertrophic marker gene expression, ANP, along with MCIP, which is an indicator of calcineurin activation (Fig. 4D). In contrast, expression of TrpC4β at similar levels had no effect on cell size or gene expression.

**TrpC4 Is Downstream of PLCβ1b in Hypertrophic Signaling.** We next examined whether TrpC4α was required for hypertrophic signaling downstream of PLCβ1b. NRVM were treated with adenovirus-expressing FLAG-PLCβ1b for 24 hours. This resulted in increases in cell size, as well as in the expression of the hypertrophic marker genes, ANP and...
Coexpression of dn-TrpC4 [Ad-myc-dn-TrpC4 (Schindl et al., 2008; Wu et al., 2010)] inhibited these PLCβ1b-induced responses, pointing to a role for TrpC4 in hypertrophic responses downstream of PLCβ1b (Fig. 5, A–E). Our previous studies have demonstrated that hypertrophy initiated by activation of Gq-coupled α1-adrenergic receptors [50 μmol/l phenylephrine plus 1 μmol/l propranolol, 24 hours] can be partially inhibited by inhibiting PLCβ1b selectively (Filtz et al., 2009). In the current studies, α1-adrenergic receptor-mediated hypertrophy was also partially inhibited by dn-TrpC4 (Fig. 5, F and G). Hypertrophy initiated by growth factors does not involve either Gq or PLCβ1b (Filtz et al., 2009). To ensure that the observed inhibitory effect of dn-TrpC4 on hypertrophic responses reflected a specific antihypertrophic action rather than general cytotoxicity, we examined the effect of dn-TrpC4 on hypertrophic responses caused by fetal bovine serum (FBS). FBS-mediated hypertrophy was not inhibited by dn-TrpC4 (Fig. 5H). Thus, TrpC4 is involved in hypertrophic signaling pathways downstream of Gq, pathways that are thought to reflect pathologic hypertrophy specifically.

Discussion

The current studies provide evidence that TrpC4α, but not the closely related TrpC4β, is involved in pathways downstream of
PLC\(\beta\)1b and contributes to cellular hypertrophic responses. PLC\(\beta\)1b is an immediate effector of signaling responses downstream of Gq-coupled receptors and is required for Gq-initiated cardiomyocyte hypertrophy (Filtz et al., 2009). PLC\(\beta\)1b expression and activity are elevated in diseased myocardium from humans, rats, mice, and sheep, and, furthermore, activity increases with disease progression (Woodcock et al., 2009). Thus, PLC\(\beta\)1b and its downstream effectors, including TrpC4\(\alpha\), may contribute to disease. TrpC4\(\alpha\) differs from the other splice variant, TrpC4\(\beta\), only in the inclusion of an 84–amino-acid sequence located close to, but not at, the extreme C-terminal end of the protein (Fig. 2A). Despite the apparently small sequence difference between the two splice variants, we found that TrpC4\(\alpha\), but not TrpC4\(\beta\), associated with both PLC\(\beta\)1b and Shank3 (Fig. 2) and translocated in response to PLC\(\beta\)1b (Fig. 3). Furthermore, TrpC4\(\alpha\), but not TrpC4\(\beta\), was sufficient to cause cardiomyocyte hypertrophy (Fig. 4). The TrpC4\(\alpha\)-specific sequence binds PIP\(_2\) (Otsuguro et al., 2008), the substrate of PLC\(\beta\)1b. Many of the TrpC family members require PIP\(_2\) for channel activity (Rohacs, 2007; Lemonnier et al., 2008), but TrpC4\(\alpha\) has the unique property of being negatively regulated by PIP\(_2\) (Otsuguro et al., 2008; Zhang et al., 2013).

TrpC4\(\alpha\), but not TrpC4\(\beta\), moved to the light membrane fraction in the presence of heightened PLC\(\beta\)1b (Fig. 3). PLC\(\beta\)1b itself and its substrate PIP\(_2\) are localized in this light membrane fraction (Morris et al., 2006). This copositioning of PLC\(\beta\)1b close to PIP\(_2\) is facilitated by its association with the scaffolding protein, Shank3, also present in the light membrane fraction (Grubb et al., 2011). In addition to binding PLC\(\beta\)1b, Shank3 has the capacity to bind to Homer family members, and in cardiomyocytes Homer1c has been shown to associate with the PLC\(\beta\)1b/Shank3 complex (Grubb et al., 2012). Homer1c has the capacity to bind any of the TrpC family and potentially could cross-link any TrpC to Shank3 (Kreienkamp, 2008). This lack of subtype specificity means that Homer cross-linking cannot explain the specific interaction between TrpC4\(\alpha\) and the PLC\(\beta\)1b/Shank3 complex.

Although the Homer-interaction domain is common to all

---

**Fig. 4.** TrpC4\(\alpha\), but not TrpC4\(\beta\), causes hypertrophy of NRVM. (A) Expression of myc-tagged TrpC4\(\alpha\) and TrpC4\(\beta\), Western blot using anti-myc antibody. Mol. wt. are indicated. (B) Upper panel, phalloidin (AlexaFluor-488 phalloidin; Life Technologies) myofilament staining of NRVM-expressing myc-TrpC4\(\alpha\) or myc-TrpC4\(\beta\) for 24 hours. Lower panel, phase-contrast images. (C) NRVM were infected with Ad-myc-TrpC4\(\alpha\) or Ad-myc-TrpC4\(\beta\), and hypertrophy was assessed by cell area and from protein/DNA ratios. Values shown are mean ± S.E.M., n = 6. *P < 0.05 relative to control. (D) Expression of TrpC4\(\alpha\), but not TrpC4\(\beta\), increased mRNA expression of ANP and MCIP. Values shown are ANP or MCIP, 2\(^{-\Delta\Delta CT}\) relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mean ± S.E.M., n = 4. †P < 0.01 relative to control. Open bars, control; black bars, TrpC4\(\alpha\); gray bars, TrpC4\(\beta\). IB, immunoblot.

---

**Fig. 5.** PLC\(\beta\)1b-induced hypertrophy is inhibited by dn-TrpC4. (A–E) NRVM were treated with Ad-FLAG-PLC\(\beta\)1b together with Ad-myc-dn-TrpC4 (Ad-dn-C4) or control virus (Ad-con) for 24 hours. (A) Representative images. (B) Cell area (\(\mu\)m\(^2\)). (C) Protein/DNA ratio. (D) ANP expression (2\(^{-\Delta\Delta CT}\) relative to glyceraldehyde-3-phosphate dehydrogenase) and (E) MCIP expression (2\(^{-\Delta\Delta CT}\) relative to glyceraldehyde-3-phosphate dehydrogenase) were measured. (F and G) NRVM were treated with phenylephrine (50 \(\mu\)mol/l) plus propranolol (PE; 1 \(\mu\)mol/l) or together with Ad-myc-dn-TrpC (ad-dn-C4) for 24 hours, and cell area was measured. (H) NRVM was treated with FBS (5%) for 24 hours, and the cell area was quantified. Values shown are mean ± S.E.M., n = 6. *P < 0.01 relative to control. †P < 0.01 relative to PLC\(\beta\)1b or PE.
TrpC, the C-terminal TTRL sequence, which is an appropriate ligand for the PDZ domain of Shank3, is exclusive to TrpC4 and TrpC5 (Beck et al., 2013). Thus, these are the only TrpC family members likely to bind Shank3 directly, that is, independently of Homer (Suh et al., 2001; Goel et al., 2005). TrpC4α, TrpC4β, and TrpC5 all have the same C-terminal TTRL motif, and therefore have identical capacities to bind to the PDZ domain of Shank3 similarly. Thus, a PDZ interaction between the TrpC and the PDZ domain of Shank3 cannot readily explain the specific interaction with TrpC4α. On this basis, another mechanism is required to explain the observed splice variant specificity of the TrpC4α/PLCβ1b/Shank3 association. Possibly, the binding to PI2P by the splice variant–specific sequence of TrpC4α (Putney, 2007; Zhang et al., 2013) is critical in localizing TrpC4α close to Shank3 and allowing binding to occur, either directly by PDZ binding or indirectly via interactions with Homer1c, or possibly by both mechanisms.

Localized Ca²⁺ responses are critical for cardiomyocyte hypertrophy, by providing the Ca²⁺ required for calcineurin activation and the initiation of downstream transcriptional responses (Goonasekera and Molkentin, 2012). Recent studies have demonstrated that these hypertrophic Ca²⁺ responses involve TrpC, in addition to a subset of L-type Ca²⁺ channels that are localized to light membrane fractions (Gao et al., 2012). Our studies showed that TrpC4α alone is sufficient to cause hypertrophy of cardiomyocytes in isolation (Fig. 4) and that dn-TrpC4 prevented hypertrophy caused by PLCβ1b (Fig. 5, A–E) and substantially reduced hypertrophy downstream of α1-adrenergic receptor activation (Fig. 5, F and G). It is generally accepted that inhibitors targeting early signaling intermediates should show specificity for individual pathways, whereas targeting downstream responses will target hypertrophy more broadly (Zhang et al., 2011). As TrpC4α is involved early in Gq/PLCβ1b-initiated hypertrophic signaling pathways, inhibition would be expected to be exclusive to these pathways. In keeping with this, dn-TrpC4 did not inhibit FBS-mediated hypertrophy, which involves pathways downstream of growth factor receptors and is independent of Gq and PLCβ1b (Simpson et al., 1982; Filtz et al., 2009; Mailet et al., 2013). TrpC4 is more highly expressed in adult myocardium than in the neonate (Jiang et al., 2014) and would also be expected to contribute to hypertrophic responses in vivo when these involve Gq. Hypertrophic responses caused by pressure overload or by infusion of angiotensin II in vivo require activation of Gq (Akhter et al., 1998; Dorn and Brown, 1999; Wetschureck et al., 2001). In keeping with our findings, previous studies have reported that expression of dn-TrpC4 reduced hypertrophy in both of these in vivo models (Wu et al., 2010).

Ascribing cardiomyocyte responses to specific TrpC subtypes has been challenging for a number of reasons. The channels have considerable structural similarity (Toko et al., 2007; Eder and Molkentin, 2011) and similar channel activity (Plant and Schaefer, 2005; Abramowitz and Birnbaumer, 2009), and are expressed at relatively low level in cardiomyocytes (Jang et al., 2012). TrpC channels form complexes that often include more than one subtype (Abramowitz and Birnbaumer, 2009), which means that overexpression or inhibition of a particular subtype may influence responses of other family members. There may also be some redundancy between the family members, whereby deletion or overexpression of one subtype results in compensation by other members, especially members of the same TrpC subfamily. These specificity issues are of less concern in the current study because overexpressing TrpC4α, but not TrpC4β, promoted hypertrophy, even though both interact similarly with TrpC1 and TrpC5 (Cioffi et al., 2012).

In the current study, we provide evidence that TrpC4 contributes to responses downstream of PLCβ1b by mechanisms that are entirely dependent on the 84–amino-acid sequence specific to the TrpC4α splice variant (Figs. 2A and 4). PI2P binding is the essential feature of TrpC4α that distinguishes it from TrpC4β in relation to its involvement in cardiomyocyte hypertrophy. As demonstrated previously (Morris et al., 2006), PI2P is located in light lipid raft fractions along with PLCβ1b, and the importance of the PI2P binding domain might be in optimizing the localization of TrpC4α in close proximity to PLCβ1b. The other possibility is that the channel is activated directly by PI2P hydrolysis resulting from PLCβ1b activation, as has been suggested in other cell types (Zhang et al., 2013). This intriguing possibility requires further investigation. We conclude that cardiomyocyte hypertrophy downstream of Gq/PLCβ1b involves specifically the higher mol. wt. splice variant of TrpC4, TrpC4α.

Acknowledgments

The authors thank Monash Medical Imaging for use of the confocal microscope and Dr. J. Putney (Research Triangle Park, NC) for the TrpC4α and TrpC4β constructs.

Authorship Contributions

Conducted experiments: Cooley, Grubb, Luo.

Wrote or contributed to the writing of the manuscript: Woodcock.

References


Gómez AM, Ruiz-Hurtado G, Benitah JP, and Domínguez-Rodríguez A (2013) Ca(2⁺)/CaM–PLCβ1b activation, as has been suggested in other cell types (Zhang et al., 2013). This intriguing possibility requires further investigation. We conclude that cardiomyocyte hypertrophy downstream of Gq/PLCβ1b involves specifically the higher mol. wt. splice variant of TrpC4, TrpC4α.


Pflugers Arch 453:753-762.


Address correspondence to: Dr. Elizabeth A. Woodcock, Baker IDI Heart and Diabetes Institute, P.O. Box 6492, St. Kilda Road Central, Melbourne, 8008, Victoria, Australia. E-mail: liz.woodcock@bakeridi.edu.au