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Phospholipase Cβ1b directly binds the SH3 domain of Shank3 for targeting and activation in cardiomyocytes

David R. Grubb, Jieting Luo, Elizabeth A. Woodcock*

Molecular Cardiology Laboratory, Baker IDI Heart and Diabetes Institute, 75 Commercial Road, Melbourne, 3004, Victoria, Australia

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ABSTRACT

Phospholipase Cβ1b (PLCβ1b) is an atypical splice variant of PLCβ1 that has a C-terminal proline-rich sequence instead of the PDZ-interacting motif common to other PLCβ subtypes. PLCβ1b targets to the cardiomyocyte sarcolemma through an undefined association with the scaffolding protein Shank3. The C-terminal splice variant specific sequence of PLCβ1b bound to deletion mutants of Shank3 that included the SH3 domain, but not to constructs lacking this domain. Mutating proline residues in the extreme C-terminal region of PLCβ1b prevented the interaction between PLCβ1b and Shank3 resulting in reduced sarcolemmal localization and downstream signalling responses. We conclude that PLCβ1b activation and downstream signalling require the association of a previously unidentified C-terminal proline-rich motif with the SH3 domain of Shank3. PLCβ1b is the first confirmed protein ligand for the SH3 domain of Shank3.

1. Introduction

Phospholipase C (PLC) subtypes are early signalling enzymes that hydrolyse the plasma membrane lipid phosphatidylinositol(4,5)*bis*phosphate (PIP_2) generating inositol(1,4,5)*tris*phosphate (IP_3) and *sn*-1,2-diacylglycerol (DAG) [1]. PLCβ family members primarily mediate responses initiated by Gq-coupled receptors [2]. Gq and its associated receptors, α_1-adrenergic receptors, endothelin receptors and angiotensin II type 1 receptors are important contributors to cardiac pathology. Dilated atrial tissue from patients with valvular heart disease displays heightened expression and activity of phospholipase Cβ1b (PLCβ1b) one of the splice variants of the early signalling enzyme, PLCβ1. PLCβ1b activity correlated with increasing atrial volume in both human disease and in a mouse model of dilated cardiomyopathy, suggesting a relationship to disease progression [3]. This possibility was supported by experiments showing that expression of PLCβ1b in cardiomyocytes resulted in pathological hypertrophy and cell death [4]. Thus the heightened PLCβ1b activity characteristic of diseased myocardium, may contribute to pathology.

Members of the PLCβ family (PLCβ1-4) generally target to particular membrane regions within the cell by binding to PDZ-domain containing protein scaffolds via a C-terminal PDZ-interacting sequence present in all expressed PLCβ subtypes, except PLCβ1b [5]. PLCβ1b is unusual in that it has proline-rich motifs in its C-terminal sequence instead of the usual PDZ ligand [6–8]. On the basis of their C-terminal sequences, the two splice variants of PLCβ1 would be predicted to have different intracellular localizations [7] and in agreement with this, PLCβ1b localized to the sarcolemma and T-tubules in neonatal and adult ventricular myocytes, whereas PLCβ1a was distributed throughout the cytosol [4]. Localization clearly determines function because PLCβ1b expression in cardiomyocytes causes hypertrophy and cell death, whereas PLCβ1a is without effect [4].

The selective localization of PLCβ1b to the sarcolemma where it can access its substrate is achieved through an as yet undefined association with a multi-protein complex involving the high MW scaffolding protein, Shank3 (SH3 domain and ankyrin repeat protein 3) [9], along with TrpC4α (transient receptor potential channel canonical 4α) and Homer1c [10] [11]. Association with the Shank3 complex serves to localize PLCβ1b in close proximity not only to its substrate lipid, but also to Homer1c and TrpC4α, both of which are involved in signalling responses downstream of PLCβ1b [10,11]. Co-expression of PLCβ1b and Shank3 has not been reported

Abbreviations: NRVM, neonatal rat ventricular myocytes; PDZ, post synaptic density protein, drosophila disc large tumor suppressor, and zonula occludens-1 protein; PLC, phospholipase C; SH3, Src homology domain 3; Shank3, SH3 and multiple ankyrin repeat protein 3.

* Corresponding author. Baker IDI Heart and Diabetes Institute, PO Box 6492, Melbourne, 3004, Victoria, Australia. Fax: +61 3 85321100.
E-mail address: liz.woodcock@bakeridi.edu.au (E.A. Woodcock).

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elsewhere [12], and thus the PLCβ1b-Shank3 complex provides the potential for a relatively cardiac-specific therapeutic target. The finding that PLCβ1b, but not PLCβ1a is incorporated into the Shank3 complex identifies the C-terminal sequence of PLCβ1b as being crucial for this association [8]. However, the specific residues within the C-terminal sequence of PLCβ1b and the domain of Shank3 responsible for this interaction remained unidentified. The splice variant specific sequence of rodent PLCβ1b includes two proline rich sequences, a PPNF sequence (1160–1164) and a PPNP (1165–1168). The PPNP motif is an ideal ligand for the type 1 SH3 domain present in Shank3, which prefers PPxP, but the PPNF sequence is also a possible SH3-interacting sequence. The PPNF sequence also might bind to the EVH1 domains in Homer1c (preferred sequence PPxxF) and thus PLCβ1b might associate with Shank3 indirectly via Homer1c crosslinking. Homer1c dimerises and crosslinks Shank3 by interaction of EVH1 domains with a proline rich motif in the Shank3 C-terminal sequence (PPPTF) [13].

The PLCβ1b-Shank3 complex is specific to cardiomyocytes and may have a role in pathological responses. For this reason, defining the interactions involved in detail may provide for future drug discovery. The current study was undertaken to identify the mechanism by which PLCβ1b associates with Shank3 in cardiomyocytes.

2. Methods

2.1. 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 accord with the provision for the care and use of laboratory animals of the Australian National health and Medical Research Council. Ventricular myocytes were prepared from 1 day old Sprague–Dawley rats of either gender, as described previously [14].

2.2. Constructs and adenoviruses

Proline/alanine mutations were generated using the GeneArt® Site-Directed Mutagenesis System (Life Technologies, Carlsbad, CA) and incorporated into adenoviral vectors using Gateway® methodology (Life Technologies). VSVg-tagged Shank3 constructs were obtained from DNA 2.0 (Menlo Park CA 94025). Lipofectamine 2000 (Life Technologies) was used for transfecting HEK293 cells.

2.3. Co-immunoprecipitation and western blotting

Cells were washed with Hanks buffered salt solution (HBSS) and then lysed and harvested in buffer and precleared as described previously [9]. Anti-FLAG antibody (F1804, Sigma–Aldrich), anti-Shank3 (Ab15302 Millipore) or anti-VSVg (V4888 Sigma–Aldrich) antibody was subsequently added at 5 μL/mL of extract, followed by Protein A or Protein G Sepharose as described [9] Proteins were separated by SDS-PAGE using gradient 7%–15% gels and were transferred to PVDF membranes for immunobloting. Antibodies dilutions used; Shank3 1/2000, FLAG 1/1000, VSVg 1/5000. All blots were developed using HRP-conjugated secondary antibodies and images were quantified using a GelDoc XRS + system (BioRad) using ImageLab 2.0.1 software.

2.4. Measurement of PLC activity and indices of hypertrophy in NRVM

These studies were performed exactly as described previously [9]. For measurement of atrial natriuretic peptide (ANP) the following primers were used: GAPDH (reference gene) 5′ atgactccactcacggcaaat, 3′ tcccattctcggccttgac; ANP 5′ atctgccctctt-gaaaagca, 3′ acacaccacaagggcttagg.

2.5. Confocal imaging

NRVM expressing FLAG-tagged PLCβ1b mutant construct were plated onto laminin-coated dishes and fixed with PFA (4% w/v paraformaldehyde, 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 PBS, and incubated with anti-FLAG and anti-Shank3 antibodies, both diluted in PBA (PBS + BSA 1% w/v). Plates were then further washed in PBS, and incubated with anti-mouse-AlexaFluor-488 and anti-rabbit-AlexaFluor-546 (both from Life Technologies). The samples were washed in PBS, and mounted using Vectashield mounting media (Abacus ALS, Australia). Images were captured using a Zeiss Meta-510 LSM (excitation 488 nm and 543 nm; emission at 519 nm and 573 nm) and analysed for red/green pixel co-localisation using Image-Pro plus 6.0 (Media Cybernetics, USA.) after background subtraction.

3. Results

3.1. The SH3 domain of Shank3 is essential for interaction with the C-terminal sequence of PLCβ1b

PLCβ1b via its splice variant specific C-terminal 32 amino acid sequence associates with a Shank3 complex at the cardiac sarcolemma [9]. The Shank3 molecule incorporates a number of domains involved in protein–protein interactions, as shown in Fig. 1A. To identify which of these is critical for association with the C-terminal

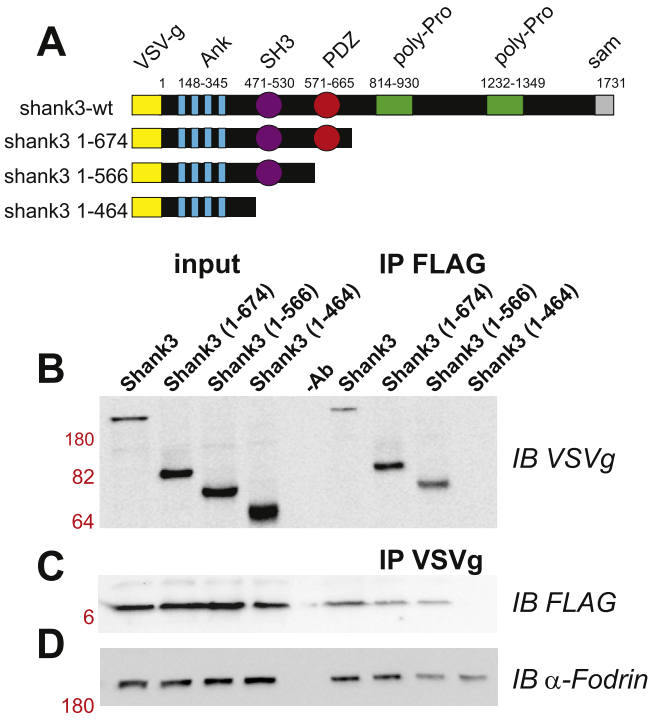

Fig. 1. *The C-terminal sequence of PLCβ1b binds to the SH3 domain of Shank3.* A. Diagram showing the domain structure of Shank3 and the 3 deletion mutants used in the current studies. Residue numbers are based on the rodent sequence. B-D. VSVg-tagged Shank3 or Shank3 deletion mutants were expressed in HEK-293 cells along with lyn-FLAG-PLCβ1b-CT. B. Extracts were prepared, immunoprecipitated with anti-FLAG antibodies and probed with anti-VSVg antibodies. C Extracts were precipitated with anti-VSVg antibodies and blotted with anti-FLAG antibody. D. Extracts were precipitated with anti-VSVg antibody and blotted with antibody to α-fodrin (positive control). The experiments were performed 3 times with similar findings.

sequence of PLCβ1b we used domain deletion mutants of Shank3: VSVg-tagged full length Shank3, Shank3(1–674) incorporating the ankyrin repeats, SH3 and PDZ domains, but lacking the C-terminal proline rich sequence that binds Homers, Shank3(1–566) including the ankyrin repeats and the SH3 domain and Shank3(1–464) including only the ankyrin repeat domain [15] (Fig. 1A). The splice variant specific C-terminal sequence of PLCβ1b was expressed as a fusion protein including a membrane-targeting sequence from the protein kinase lyn (lyn 1–10) and the FLAG epitope (lyn-FLAG-PLCβ1b-CT) [4]. Lyn-FLAG-PLCβ1b-CT was expressed in HEK-293 cells together with VSVg-tagged Shank3 or Shank3-C-terminal deletion mutant (Fig. 1A). Extracts were prepared and immunoprecipitated with anti-FLAG antibody. Lyn-FLAG-PLCβ1b-CT co-immunoprecipitated with Shank3 (full length), Shank3(1–674) and Shank3(1–566) but not with Shank3(1–466), as shown by western blotting with anti-VSVg antibodies (Fig. 1B). When extracts were immunoprecipitated with anti-VSVg antibodies, VSVg-tagged Shank3, Shank3(1–674) and Shank3(1–566) co-immunoprecipitated with lyn-FLAG-PLCβ1b, Shank3(1–466) did not (Fig. 1C). As a positive control we examined the ability of α-fodrin, which associates with the ankyrin repeat sequences of Shank3, to bind Shank3(1–466). α-Fodrin immunoprecipitated with Shank3(1–466), as well all other Shank3 constructs (Fig. 1D), providing a positive control for the study. The finding that lyn-FLAG-PLCβ1b-CT immunoprecipitated with Shank3(1–674) and (1–566) in addition to the full length protein shows that the Homer-binding sequence of Shank3 (814–930) is not required. This argues against the possibility that PLCβ1b binds to Shank3 via Homer1c crosslinking. Instead, by showing binding to Shank3(1–566) but not Shank3(1–464), the data implicate the SH3 domain of Shank3 as the binding site for the C-terminal region of PLCβ1b.

3.2. PLCβ1b-Shank3 binding involves a proline rich sequence

Studies described above localized the interaction between the C-terminal 32 residue sequence of PLCβ1b to the SH3 domain of Shank3, a motif that binds to proline rich sequences [9]. The C-terminal of PLCβ1b includes an extended proline-rich region (1160–1168) (Fig. 2A) and we next tested the proline residues within this region for their involvement in the association of PLCβ1b with Shank3. FLAG tagged PLCβ1b-WT or the Pro/Ala mutants PLCβ1b^{P1160,1161A} or PLCβ1b^{P1165,1166A} were expressed in HEK-293 cells together with VSVg-tagged full length Shank3. Extracts were prepared, immunoprecipitated with anti-FLAG antibody and subsequently immunoblotted with VSVg antibody to detect bound Shank3. As shown in Fig. 2B, VSVg-tagged Shank3 immunoprecipitated with FLAG-PLCβ1b-WT and to a lesser extent with FLAG-PLCβ1b^{P1160,1161A}. In contrast, VSVg-Shank3 showed no co-immunoprecipitation with FLAG-PLCβ1b^{P1165,1166A}. Immunoblotting with anti-FLAG antibodies showed similar levels of immunoprecipitation for all PLCβ1b constructs. A similar set of studies was conducted where extracts were immunoprecipitated with anti-VSVg antibodies and subsequently immunoblotted with anti-FLAG antibodies. As shown in Fig. 2C, FLAG-PLCβ1b-WT and FLAG-PLCβ1b^{P1160,1161A} co-immunoprecipitated with anti-VSVg antibodies, but FLAG-PLCβ1b^{P1165,1166A} did not. VSVg-Shank3 was similarly precipitated by anti-VSVg antibodies in all treatment groups. These data identify the C-terminal proline residues P^{1165} and P^{1166} as being critical for the interaction between PLCβ1b and Shank3.

3.3. PLCβ1b activity in NRVM requires a C-terminal proline-rich sequence

The association between PLCβ1b and Shank3 in neonatal rat ventricular myocytes (NRVM) is required for PLC activation and for

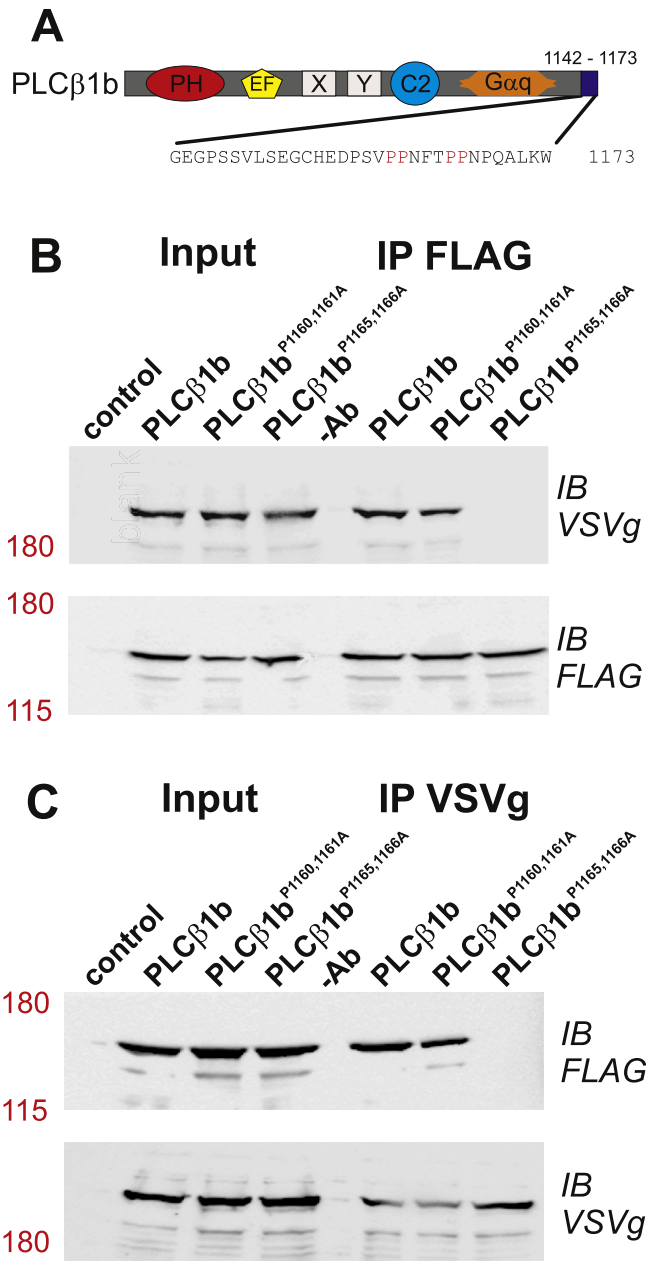

Fig. 2. *PLCβ1b wild type and PLCβ1b^{P1160,1161A} but not FLAG-PLCβ1b^{P1165,1166A} immunoprecipitates with Shank3.* A. The sequence of the splice variant specific C-terminal sequence of PLCβ1b. Proline residues mutated to alanine are shown in red. Extracts from HEK-293 cells transfected with control, FLAG-PLCβ1b-WT, FLAG-PLCβ1b^{P1160,1161A} or FLAG-PLCβ1b^{P1165,1166A} were immunoprecipitated with either B. Anti-FLAG antibody and probed with anti-VSVg (upper panel) and anti-FLAG antibodies (lower panel), or, C. Anti-VSVg antibody and probed with anti-FLAG antibody (*upper panel*) and anti-VSVg antibodies (*lower panel*). The experiments were performed 3 times with similar findings.

downstream hypertrophic responses [4,8] We next examined the abilities of the Pro/Ala mutants (FLAG-PLCβ1b^{P1160,1161A} or FLAG-PLCβ1b^{P1165,1166A}) to localize to the sarcolemma, to increase PLC activity and to cause cardiomyocyte hypertrophy. FLAG-tagged PLCβ1b-WT, PLCβ1b^{P1160,1161A} or PLCβ1b^{P1165,1166A} were expressed in NRVM using adenoviral delivery. PLCβ1b-WT localized to the sarcolemma of neonatal rat cardiomyocytes and co-localised with Shank3, as reported previously [8]. Site-directed mutagenesis of P^{1160}/P^{1161} to alanine reduced co-localisation of PLCβ1b with endogenous Shank3 (Fig. 3A). In contrast, PLCβ1b^{P1165,1166A} did not

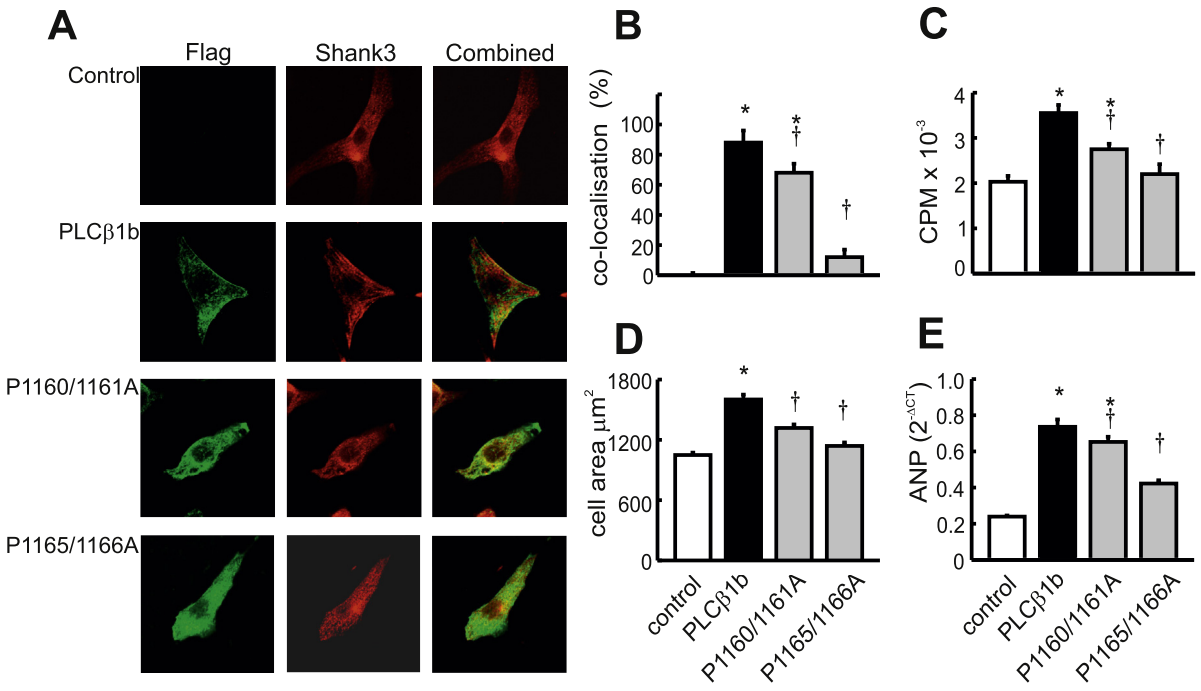

Fig. 3. *Co-localisation with Shank3 requires $P^{1156,1166}$ of PLCβ1b.* Adenoviruses expressing control, FLAG-PLCβ1b-WT, FLAG-PLCβ1b^{P1160,1161A} or FLAG-PLCβ1b^{P1165,1166A} were expressed in NRVM. A. Co-localization of PLCβ1b, and PLCβ1b-P/A mutants and endogenous Shank3 in NRVM. Immunofluorescence of treated NRVM was performed using anti-FLAG monoclonal antibodies followed by anti-mouse-AlexaFluor-488 (green) and anti-Shank3 poly-clonal antibodies followed by anti-rabbit-AlexaFluor-546 (red). Images are typical of those from 4 different experiments. Images capture using a Zeiss Meta-510 (ex 488/520 nm; em. 543/573 nm). B. Co-localization of control, FLAG-PLCβ1b-WT, FLAG-PLCβ1b^{P1160,1161A} and FLAG-PLCβ1b^{P1165,1166A} with endogenous Shank3 in NRVM assessed as red/green pixel co-localization. Values shown are mean ± SEM, n = 30. *p < 0.05 relative to control. †p < 0.05 relative to PLCβ1b-WT. C. Phospholipase C (PLC) activity in NRVM as indicated. Values shown are mean ± SEM, n = 6. *p < 0.01 relative to control. †p < 0.01 relative to PLCβ1b-WT. D. Cell area in NRVM expressing PLCβ1b-WT or mutants, as indicated. E. Expression of the hypertrophic marker ANP in NRVM treated as indicated. Data expressed as $2^{-\Delta CT}$ of the reference gene (GAPDH), and shown as mean ± SEM, n = 6. *p < 0.01 relative to control. †p < 0.01 relative to PLCβ1b-WT.

show clear membrane localization (Fig. 3A) nor did this construct show a high degree of co-localisation with endogenous Shank3 (Fig. 3B). PLC activation requires localization at the sarcolemma in order to access its substrate PIP_2. Heightened expression of PLCβ1b-WT increased PLC activity in NRVM [4], whereas expression of PLCβ1b^{P1165,1166A} did not significantly alter PLC activity. Increased PLC activity was observed following expression of PLCβ1b^{P1160,1161A} but was lower than with PLCβ1b-WT (Fig. 3C). As reported previously [4], expression of PLCβ1b-WT induced hypertrophy of NRVM, indicated in the current study by increased cell surface area (Fig. 3D) and increased expression of the hypertrophic marker gene atrial natriuretic peptide (ANP) (Fig. 3E). Expressing PLCβ1b$^{P1160,/1161A}$ had similar effects to PLCβ1b-WT although it was less effective for all responses. Expressing of PLCβ1b^{P1165,1166A} did not increase PLC activity and did not alter cell size (Fig. 3C&D). A small increase in ANP expression was observed (Fig. 3E). Thus removal of two of the C-terminal proline residues, those that are part of the PPNP sequence (1165–1168) interferes with the functioning of PLCβ1b in cardiomyocytes.

4. Discussion

The C-terminal sequence of PLCβ family members is critical for targeting the enzyme to particular membranes localizations where it can be activated. For most PLCβ subtypes, membrane targeting involves a subtype-specific C-terminal PDZ-interacting sequence that associates with the PDZ domain of a membrane associated protein scaffold [5]. By associating with different PDZ proteins, PLCβ family members can have different physiological functions even within the same cell type. PLCβ3 by PDZ association forms a ternary complex involving PDZ domain-containing 1 (PDZK1) and somatostatin receptors and is solely responsible for somatostatin signalling in HEK-293 cells, even though PLCβ1 is also expressed [16]. In the same cell type, both PLCβ1a and PLCβ3 associate with the cell polarity proteins, Par3 and Par6, whereas PLCβ2 and PLCβ4 do not [17]. In post-synaptic density regions of central neurons, the C-terminal sequence of PLCβ3, but not that of other PLCβ subtypes, interacts with the PDZ domain of Shank2, bringing it in close proximity to Homer1b, which, in turn, links to metabatropic glutamate and IP_3-receptors [18]. In HEK-293 cells as well as in brain tissue, PLCβ1a and PLCβ2 associate with the first PDZ domain of the scaffold protein NHERF1 (sodium–hydrogen exchanger regulatory factor 1) and share this association with TrpC4 and TrpC5, both of which also have C-terminal PDZ ligands. This scaffolding promotes the regulation of TrpC4/5 by PLC activation [19]. Thus highly specific PDZ interactions are ultimately responsible for the specificity of signalling downstream of PLCβ family members. Interestingly, PLCβ1b and PLCβ3 both associate with Shank family members, but use different mechanisms to achieve this. PLCβ1b binds to the SH3 domain of Shank3 whilst PLCβ3 associates with Shank2 by PDZ interaction [18]. In either case, the interaction with Shank facilitates Homer interaction and thereby promotes further crosslinking with other signalling intermediates [10,18]. Thus the overall outcome appears to be similar whichever mechanism is used for localization.

The Shank family of high MW scaffolding proteins have mostly been studied in relation to the functioning of the post synaptic density region of central neurons, especially metabotropic glutamate neurones [20,21]. Shank proteins homodimerise through their C-terminal sterile alpha motif (SAM) domains and are cross-linked by Homer proteins via EVH1-proline rich domain interactions [13]. Together Shanks and Homers form three dimensional networks that serve to organise and facilitate/optimise the

functioning of the post-synaptic region of the neuron. Shank family members bind to cytoskeletal proteins via ankyrin repeat domains in the N-terminal region (Fig. 1A) [12,22]. In addition, a number of factors that associate with the PDZ domains of Shank family members have been identified [23]. These include guanylate kinase associated protein (GKAP) [24], ProSaP (Shank) interacting protein 1 (ProSapIP1) and ProsapIP2 [25,26], the GluR1 subunit of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors [27]. In hippocampal neurons, the α subunit of the voltage-regulated Ca^{2+} channel $Ca_V1.3$, but not the closely related $Ca_V1.2$, binds to Shank via PDZ interaction [28,29] and in epithelial cells, Shank3 regulates tyrosine kinase cascades by PDZ-dependent binding of the tyrosine kinase Ret9 [30]. In marked contrast to the plethora of proteins shown to associate with the PDZ domains of Shank family members, few ligands of the SH3 domain have been identified. The SH3 domain of Shank3 is a type 1 SH3 domain with a preference for PPxP sequences such as found in the C-terminal tail of PLCβ1b [31], and the current studies have verified interaction between this sequence and the SH3 domain of Shank3. In addition to our studies in cardiomyocytes, the suggestion has been made that PPxP sequences present in the C terminus of $Ca_V1.3\alpha$ can bind to the Shank SH3 domain in addition to the above mentioned PDZ interaction [28,29].

The interaction between PLCβ1b C-terminal sequences and the SH3 domain of Shank3 appears to be unique to cardiomyocytes. Both PLCβ1b and Shank3 are expressed only in a limited number of cell types and, to date cardiomyocytes are the only cell type where co-expression has been reported [9]. On this basis, inhibiting this proline-rich/SH3 interaction provides a high specificity target for drug development.

Authorship

DRG designed and performed most of the studies described. JTL prepared and maintained the NRVM and HEK-293 cultures and performed some of the western blots. EAW planned the study and wrote the manuscript.

Conflicts of interest

None of the authors has any conflicts to disclose.

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