

## A Canonical EF-Loop Directs Ca<sup>2+</sup>-Sensitivity in Phospholipase C- $\eta$ 2

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### ABSTRACT

Phospholipase C- $\eta$  (PLC $\eta$ ) enzymes are a class of phosphatidylinositol 4,5-bisphosphate-hydrolyzing enzymes involved in intracellular signaling. PLC $\eta$ 2 can sense Ca<sup>2+</sup> (stimulated by  $\sim$ 1  $\mu$ M free Ca<sup>2+</sup>) suggesting that it can amplify transient Ca<sup>2+</sup> signals. PLC $\eta$  enzymes possess an EF-hand domain composed of two EF-loops; a canonical 12-residue loop (EF-loop 1) and a non-canonical 13-residue loop (EF-loop 2). Ca<sup>2+</sup>-binding to synthetic peptides corresponding to EF-loops 1 and 2 of PLC $\eta$ 2 and EF-loop 1 of calmodulin (as a control) was examined by 2D-[<sup>1</sup>H, <sup>1</sup>H] TOCSY NMR. Both PLC $\eta$ 2 EF-loop peptides bound Ca<sup>2+</sup> in a similar manner to that of the canonical calmodulin EF-loop 1, particularly at their N-terminus. A molecular model of the PLC $\eta$ 2 EF-hand domain, constructed based upon the structure of calmodulin, suggested both EF-loops may participate in Ca<sup>2+</sup>-binding. To determine whether the EF-hand is responsible for Ca<sup>2+</sup>-sensing, inositol phosphate accumulation was measured in COS7 cells transiently expressing wild-type or mutant PLC $\eta$ 2 proteins. Addition of 70  $\mu$ M monensin (a Na<sup>+</sup>/H<sup>+</sup> antiporter that increases intracellular Ca<sup>2+</sup>) induced a 4- to 7-fold increase in wild-type PLC $\eta$ 2 activity. In permeabilized cells, PLC $\eta$ 2 exhibited a  $\sim$ 4-fold increase in activity in the presence of 1  $\mu$ M free Ca<sup>2+</sup>. The D256A (EF-loop 1) mutant exhibited a  $\sim$ 10-fold reduction in Ca<sup>2+</sup>-sensitivity and was not activated by monensin, highlighting the involvement of EF-loop 1 in Ca<sup>2+</sup>-sensing. Involvement of EF-loop 2 was examined using D292A, H296A, Q297A, and E304A mutants. Interestingly, the monensin responses and Ca<sup>2+</sup>-sensitivities were largely unaffected by the mutations, indicating that the non-canonical EF-loop 2 is not involved in Ca<sup>2+</sup>-sensing. *J. Cell. Biochem.* 115: 557–565, 2014. © 2013 Wiley Periodicals, Inc.

**KEY WORDS:** CALCIUM; CELL SIGNALING; COMPARATIVE MODELING; EF-HAND; PHOSPHOLIPASE C; SIGNAL TRANSDUCTION

The intracellular movement of Ca<sup>2+</sup> in response to the chemical messenger inositol 1,4,5-trisphosphate (IP<sub>3</sub>) triggers a range of physiological processes. Upon activation, Ca<sup>2+</sup> is released from intracellular stores into the cytoplasm whereby the intracellular free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) rises from  $\sim$ 10<sup>-7</sup> M to  $\sim$ 10<sup>-6</sup> M [Berridge et al., 2000]. Generally, this increase in [Ca<sup>2+</sup>]<sub>i</sub> results in the activation of effector proteins, where the binding of Ca<sup>2+</sup> alters the proteins' conformation. This is then translated, often through a complex of proteins, into a physiological event. Many of these Ca<sup>2+</sup>-

effector proteins contain homologous Ca<sup>2+</sup>-binding domains termed EF-hands [Strynadka and James, 1989; Gifford et al., 2007].

Structurally, EF-hand domains consist of one or more pairs of EF-loops, each of which constitutes a structurally independent, functional domain [Drabikowski et al., 1982]. Each EF-loop (usually but not always) binds Ca<sup>2+</sup> and links two  $\alpha$ -helices positioned roughly perpendicular to one another forming a helix-loop-helix structure. Within the EF-hand these two motifs are stacked against one another in a face-to-face manner. This structure is further

Abbreviations: [Ca<sup>2+</sup>]<sub>i</sub>, intracellular free Ca<sup>2+</sup> concentration; CaM, calmodulin; DAG, 1,2-diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; NCS-1, neuronal calcium sensor-1; NOESY, nuclear Overhauser effect spectroscopy; PH, pleckstrin homology; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; TOCSY, total correlation spectroscopy.

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stabilized by a short antiparallel  $\beta$ -sheet formed between the pairs' EF-loops [Grabarek, 2006]. The structural and metal ion-binding properties of these domains are reviewed by Gifford et al. (2007). Examples of  $\text{Ca}^{2+}$ -responsive effector proteins that contain EF-hand domains include calmodulin (CaM; Vetter and Leclerc, 2003), neuronal calcium sensor-1 (NCS-1; Burgoyne, 2004) and troponin C [Parmacek and Leiden, 1991]. EF-hand (or EF-hand-like) domains are also present in the mammalian phosphatidylinositol 4,5-bisphosphate ( $\text{PIP}_2$ )-specific phospholipase C (PLC) enzymes. Once activated these enzymes catalyze the cleavage of  $\text{PIP}_2$  to generate the secondary messengers 1,2-diaclyglycerol (DAG) and  $\text{IP}_3$ . DAG activates protein kinase C whilst  $\text{IP}_3$  triggers the release of  $\text{Ca}^{2+}$  from intracellular stores. The EF-hand domains of most of these enzymes have limited or no ability to bind  $\text{Ca}^{2+}$  [Bairoch and Cox, 1990]. A key exception to this is PLC $\zeta$ , which is responsible for  $\text{Ca}^{2+}$  oscillations in fertilized oocytes and exhibits a high sensitivity to  $\text{Ca}^{2+}$  mediated through its EF-hand domain [Kouchi et al., 2005]. Also it appears that the EF-hand domain of PLC $\delta$ 1 is functionally important and regulates binding of the pleckstrin homology (PH) domain to  $\text{PIP}_2$  in a  $\text{Ca}^{2+}$ -dependent manner [Yamamoto et al., 1999].

The most recent class of mammalian PLC enzyme to be identified - the PLC $\eta$  enzymes-are most closely related to the PLC $\delta$  class [Hwang et al., 2005; Nakahara et al., 2005; Stewart et al., 2005; Zhou et al., 2005]. Two putative PLC $\eta$  enzymes are present in humans and mice, PLC $\eta$ 1 and PLC $\eta$ 2 [Stewart et al., 2005]. Both catalyze hydrolysis of  $\text{PIP}_2$  [Hwang et al., 2005; Nakahara et al., 2005; Zhou et al., 2005], which suggests that these enzymes, like other  $\text{PIP}_2$ -specific PLCs, are involved in the production of DAG and  $\text{IP}_3$ . The cellular function of these enzymes is not entirely clear, although a recent study highlighted a potential role for PLC $\eta$ 2 in regulating neurite growth [Popovics et al., 2013]. It has become apparent that both PLC $\eta$  enzymes are activated by intracellular  $\text{Ca}^{2+}$  mobilization [Kim et al., 2011; Popovics et al., 2011], and are sensitive to changes in  $[\text{Ca}^{2+}]_i$  within the physiological range [Hwang et al., 2005; Nakahara et al., 2005]. This discovery provides a rationale for the existence of a positive-feedback loop for  $\text{Ca}^{2+}$ , whereby PLC $\eta$  enzymes can modulate further (or sustained) release of  $\text{Ca}^{2+}$  from intracellular stores through production of the secondary messenger,  $\text{IP}_3$ . In this study we demonstrate that the submicromolar  $\text{Ca}^{2+}$ -sensitivity of PLC $\eta$ 2 is directed by a functional EF-hand domain.

## MATERIALS AND METHODS

### MATERIALS

The peptides used in this study (CaM-EF1, Ac-LFDKDGDTITTKEL;  $\eta$ 2-EF1, Ac-EADKNGDGSLSIGE;  $\eta$ 2-EF2, Ac-EADTDDHQGTGTFEE) were synthesized by EZBiolab (Westfield, IN). The expression construct encoding residues 75–1,238 of mouse PLC $\eta$ 2 (isoform a; NP\_780765) in pcDNA3.1 (as used in Nakahara et al. [2005]) was a gift from Prof. Kiyoko Fukami (Tokyo University of Pharmacy and Life Science, Japan). This construct was used to synthesize D256A, D292A, H296A, Q297A, and E304A mutants using the Quikchange Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands).

### NMR STUDIES

1D and 2D [ $^1\text{H}$ ,  $^1\text{H}$ ] TOCSY (60 ms mixing time, using an MLEV-17 [Bax and Davis, 1985] sequence for spin-lock) and NOESY (500 ms mixing time) spectra were acquired at 298 K on 1 mM samples in  $\text{DMSO-d}_6$ , both in the presence and absence of 5 mM  $\text{CaCl}_2$ , on a Bruker DRX500 spectrometer, operating at 500.13 MHz for  $^1\text{H}$ . Residual water was suppressed using excitation sculpting with gradients [Hwang and Shaka, 1995]. All datasets were acquired with a spectral width of 13 ppm, using 4k data points in F2, 400 increments in F1, and the States-TPPI (time-proportional phase incrementation; Marion et al., 1989) acquisition mode. Spectra were processed with TopSpin v. 2.1, using shifted sine-bell functions for apodization in both dimensions, and Fourier-transformed using  $2k \times 2k$  data points. The residual DMSO peak at 2.51 ppm was used for calibrating all spectra. In all but one case (the NH resonance for K13 in calmodulin EF-loop 1 was not observed) complete sequential assignment was achieved using routine methods and the visualization and assignment program Sparky v. 3.111. The resulting chemical shifts for backbone protons were used to explore the effects of  $\text{Ca}^{2+}$ . The dipolar aprotic solvent  $\text{DMSO-d}_6$  was chosen because signals for labile protons such as those of the NH backbone protons, are often reduced or missing in aqueous media due to exchange with bulk water but readily observed in DMSO. Most peptides have good solubility in DMSO, and a large number of conformational studies of short peptides have been carried out in  $\text{DMSO-d}_6$  [Oliva et al., 2000], including studies on Ca-binding peptides [Gaggelli et al., 1999]. It is noteworthy that DMSO does not induce secondary structure [Yeagle et al., 2007], can act as an H-bond acceptor, but not a donor, and has a weaker tendency than water to co-ordinate to metal ions. Based on the lower dielectric constant (46.7) and the slightly lower Gutmann donicity of DMSO [Marcus, 1984], the tendency for  $\text{Ca}^{2+}$ -peptide binding is expected to be moderately increased in DMSO, compared to aqueous solutions.

### INOSITOL PHOSPHATE RELEASE ASSAYS

Plasmids encoding wild-type and mutant PLC $\eta$ 2 were transiently transfected into COS7 cells by electroporation and [ $^3\text{H}$ ]inositol phosphate release was assayed as described previously [Popovics et al., 2011]. Briefly, transfected cells were seeded onto 12-well plates. After 24 h, cells were labeled overnight with  $1 \mu\text{Ci/ml}$  myo-D- [ $^3\text{H}$ ] inositol (Perkin-Elmer, Cambridge, UK) in inositol-free DMEM (MP Biomedicals, Illkirch, France) in the absence of serum. Cells were stimulated with 0–70  $\mu\text{M}$  monensin (Sigma-Aldrich, Poole, UK) at 37°C for 3 h following addition of fresh HEPES-buffered DMEM containing 10 mM LiCl to enable accumulation of inositol phosphates. Stimulation was terminated by the removal of the medium and addition of 10 mM formic acid. The  $^3\text{H}$ -labelled inositol phosphates were isolated from the extracts using Dowex AG 1-X8 ion exchange resin (BioRad, Hemel Hempstead, UK), eluted with 1 M ammonium formate/0.1 M formic acid and quantified by liquid scintillation counting.

For experiments investigating  $\text{Ca}^{2+}$ -sensitivity, transfected cells were seeded into 15 cm diameter dishes and the phosphatidylinositol pool was labeled with  $1 \mu\text{Ci/ml}$  myo-D- [ $^3\text{H}$ ]inositol in inositol-free DMEM containing 10% fetal calf serum. Cells were trypsinized and resuspended in permeabilization buffer (20 mM PIPES, 129 mM potassium glutamate, 5 mM glucose, 5 mM ATP, 5.31 mM  $\text{MgCl}_2$ , 5 mM EGTA, 10 mM LiCl, 0.1% BSA, pH 6.6). Cells were counted and

electrically permeabilized (10 discharges of 3 kV/cm<sup>3</sup>) using a custom-made device. Varying quantities of CaCl<sub>2</sub> were added to give appropriate free calcium concentrations when in equilibrium with 5 mM EGTA at pH 6.6 [Portzehl et al., 1964]. The cell suspensions were incubated at 37°C for 2 h before addition of formic acid (10 mM final concentration). <sup>3</sup>H-labelled inositol phosphates were isolated and measured as described above. Results were normalized to counts/number of cells and expressed as fold changes relative to basal levels. All [<sup>3</sup>H]inositol phosphate release assays were performed in triplicate and the results presented are representative of multiple experiments.

## IMMUNOBLOTTING

Transfected COS7 cells were seeded onto six-well plates. Cells were scraped 48 h post-transfection in RIPA buffer containing complete protease inhibitor cocktail (Roche, Burgess Hill, UK). Cell lysates were resuspended in equal volumes of gel running buffer (125 mM Tris-HCl, 20% glycerol, 4% SDS, and 90 mM β-mercaptoethanol, pH 6.8). Samples were separated by NuPAGE gradient (4–12% Bis-Tris) gel electrophoresis using a MES buffer system (Invitrogen, Paisley, UK) then electro-blotted for 75 min at 30 mA/gel onto PVDF membrane (GE Healthcare, Hertfordshire, UK). Blots were incubated for 1 h at room temperature in blocking buffer (5% non-fat milk in Tris-buffered saline, TBS) before being incubated for 90 min in 1% non-fat milk and 0.5% Tween-20 in TBS containing either goat polyclonal anti-PLC $\eta$ 2 antibody (Santa Cruz Biotechnology, Heidelberg, Germany) or mouse monoclonal anti-β-actin antibody (Sigma-Aldrich). Blots were washed three times in TBST (TBS containing 0.5% Tween) after antibody incubations. Specific antibody binding was detected by chemiluminescence with horseradish peroxidase-conjugated secondary antibody and chemiluminescent substrate (both Thermo Fisher Scientific, Loughborough, UK) using a LAS-3000 imager (Fujifilm, Düsseldorf, Germany).

## MOLECULAR MODELING

A molecular model of the EF-hand domain of PLC $\eta$ 2 was constructed based upon the structure of calmodulin (CaM) (PDB: 3EVU) [Wang et al., 2008] using the Modeller 9v1 program [Marti-Renom et al., 2000]. Despite the relatively low sequence identity between PLC $\eta$ 2 and CaM (~30%), the alignment between template and target sequence is unambiguous for EF-loop 1 (see Fig. 1D, *HsCaMEF1* and *MmPLC $\eta$ 2*). Due to the one residue insertion into PLC $\eta$ 2 within the EF-loop 2, either of His296 and Gln297 can in principle be aligned to position “Z” depending on the positioning of the gap. To account for these two possibilities and to avoid bias, all alignment-based constraints for His296 and Gln297 were omitted during the modeling process. The resulting models were inspected manually to identify which of the two residues is a more likely candidate to ligate Ca<sup>2+</sup>.

## RESULTS

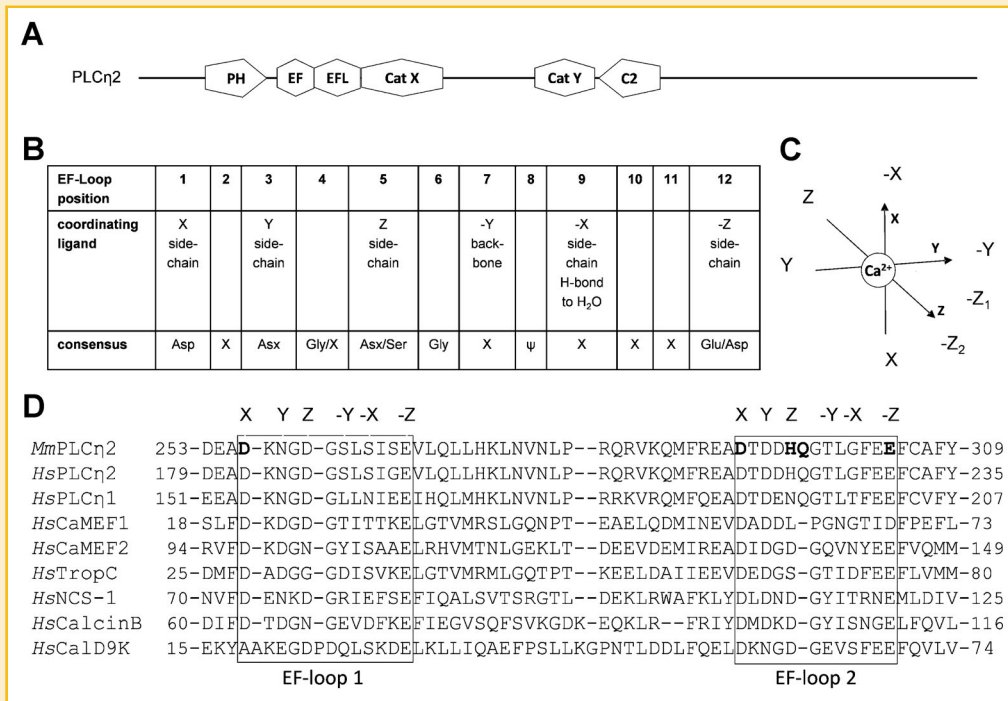
### ANALYSIS OF EF-HAND DOMAINS FROM PLC $\eta$ ENZYMES

Analysis of conserved domains from PLC $\eta$  enzymes suggest that in addition to an EF-hand-like domain, which is found in most vertebrate PLC enzymes (and generally has little or no affinity toward Ca<sup>2+</sup>), the  $\eta$ -class of phospholipase C enzymes may possess an additional functional EF-hand domain containing a pair of Ca<sup>2+</sup>-

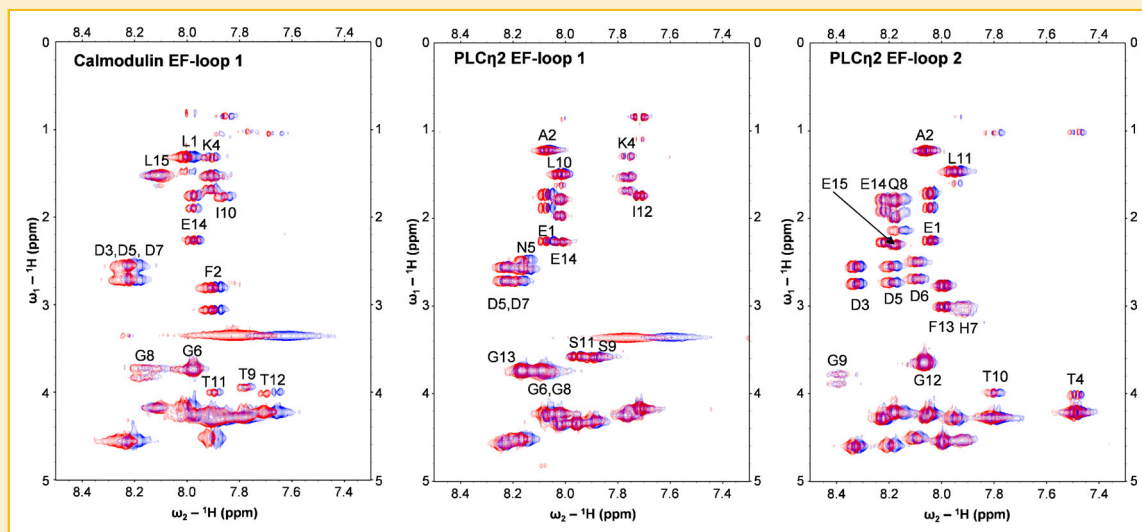
binding EF-loops (Fig. 1A). EF-loops are defined here as the structural motifs present within the EF-hand domain responsible for Ca<sup>2+</sup>-binding. Paired EF-loops are usually only found in Ca<sup>2+</sup>-effector proteins [Gifford et al., 2007]. Indeed, the sequences corresponding to these domains in both PLC $\eta$  proteins are conserved relative to the EF-hands of other proteins. Canonical EF-loops consist of 12 residues and have the consensus sequence DXB(G/X)(B/S)GX $\Psi$ XXX(E/D), where B indicates either Asn/Asp and  $\Psi$  indicates a hydrophobic residue [Gifford et al., 2007]. These loops provide 7 O-ligands (named “X,” “Y,” “Z,” “-X,” “-Y,” “-Z<sub>1</sub>,” and “-Z<sub>2</sub>”) to form a septadentate Ca<sup>2+</sup> site as illustrated in Figure 1B and C. However, the second of the two putative EF-loops in PLC $\eta$  enzymes appears to contain 13 residues, representing a novel type of non-canonical EF-loop (Fig. 1D). The sequence alignment reveals that the “extra residue” in EF-loop 2 is most likely to be either H296 or Q297 at positions 5 and 6, respectively. This 13-residue loop is conserved in all known vertebrate PLC $\eta$  enzymes (including mammals, amphibians, birds and fish; Fig. S1), suggesting that this motif is (or was) functionally important and originated (probably due to a codon insertion event) prior to the divergence of vertebrates 500 million years ago. Non-canonical loops have been identified in a number of EF-hand-containing proteins, which in some cases are able to co-ordinate to Ca<sup>2+</sup> [Grabarek, 2006; Gifford et al., 2007]. An example (human calbindin D<sub>9k</sub>) is illustrated in the alignment in Figure 1D. The first of the two EF-loops in this protein contains an additional Ala residue between what would correspond to the canonical first and second residues and a Pro residue between the fifth and sixth. Structural studies reveal that in this case the co-ordinating ligands “X,” “Y,” and “Z” are provided by the backbone carbonyl groups rather than the amino acid side-chains [Kördel et al., 1993].

### CA<sup>2+</sup>-BINDING PROPERTIES OF SYNTHETIC PLC $\eta$ 2 EF-LOOP PEPTIDES

In order to probe whether and how Ca<sup>2+</sup> may co-ordinate to the EF-loops within the EF-hand domain of PLC $\eta$ 2, we generated synthetic peptides corresponding to each of the two putative Ca<sup>2+</sup>-binding loops and as a control, the first EF-loop of CaM. NMR spectra (2D <sup>1</sup>H TOCSY and NOESY) were recorded for each of the three peptides in the presence and absence of 5 mM Ca<sup>2+</sup> (2D <sup>1</sup>H TOCSY spectra are shown in Fig. 2). The addition of a 5-fold molar excess of Ca<sup>2+</sup> to the peptides elicited significant changes in chemical shifts of the backbone NH protons of both PLC $\eta$ 2 EF-loop peptides, comparable to those observed for the CaM EF-loop 1 peptide (as summarized in Fig. 3). A titration with up to 500 mM Ca<sup>2+</sup> for loop 2 gave a dissociation constant of K<sub>D</sub> = 20 ± 4 mM in aqueous solution (Fig. S2). Since the overall magnitude of the observed effects at 5 mM Ca<sup>2+</sup> were similar for all three peptides, it is clear that Ca<sup>2+</sup> binding to all three isolated loops was, as expected, considerably weaker than to intact EF-hand motifs [Borin et al., 1989]. It can be estimated that at the concentrations used for the 2D <sup>1</sup>H NMR experiments (1 mM peptide, 5 mM Ca<sup>2+</sup>), a mixture of bound and unbound peptides was present. Only a single set of resonances was observed in either the presence or absence of Ca<sup>2+</sup> for all peptides, indicating that the systems are in fast exchange on the NMR timescale. Hence, the resonances observed in the presence of Ca<sup>2+</sup> also correspond to a mixture between peptides with and without Ca<sup>2+</sup> bound, with the magnitude of the chemical



**Fig. 1.** A: Domain structure of phospholipase C- $\eta$ 2. PH, pleckstrin homology domain; EF, EF-hand domain; EFL, EF-hand-like domain; Cat X and Cat Y, catalytic domains; C2, Ca<sup>2+</sup>-dependent membrane binding domain. B: Sequence preference of EF-loops (modified from Gifford et al., 2007). Ligands "X," "Y," "Z," "-X," "-Y," "-Z<sub>1</sub>," and "-Z<sub>2</sub>" are indicated by their position in the loop and whether co-ordination occurs via side-chain or backbone carbonyl oxygens. Ligand "-X" is a water molecule H-bonded to the side-chain. Note that residue 12 is usually a bidentate ligand (contributing both "-Z<sub>1</sub>" and "-Z<sub>2</sub>" ligands), that is, side-chain carboxylate oxygens of either Glu or Asp. In the Ca<sup>2+</sup>-bound state the ligands co-ordinate forming a pentagonal bipyramid geometry around the Ca<sup>2+</sup> ion as shown in (C). The "Y," "-Y," "Z," "-Z<sub>1</sub>," and "-Z<sub>2</sub>" ligands align along the vertices of an approximately planar pentagon with ligands "X" and "-X" in an axial position  $\sim 90^\circ$  to the other five [Strynadka and James, 1989]. D: Partial sequence alignment of EF-hand domains of PLC $\eta$  enzymes and other proteins. Each of the two EF-loops is indicated by the box. *MmPLC $\eta$ 2*, murine phospholipase C- $\eta$ 2; *HsPLC $\eta$ 2*, human phospholipase C- $\eta$ 2; *HsPLC $\eta$ 1*, human phospholipase C- $\eta$ 1; *HsCaMEF1*, human calmodulin EF-hand domain 1; *HsCaMEF2*, human calmodulin EF-hand domain 2; *HsTropCII*, human troponin C (type2); *HsNCS-1*, human neuronal calcium sensor-1; *HsCalcinB*, human calcineurin B; *HsCalD9K*, human calbindin D<sub>9K</sub>.



**Fig. 2.** Overlays of the fingerprint region of 2D-<sup>1</sup>H TOCSY NMR spectra of EF hand loop peptides in the presence (red) and absence (blue) of a 5-fold excess of Ca<sup>2+</sup> (1 mM peptide, DMSO-d<sub>6</sub>, 298 K, 500 MHz). Each set of vertically aligned peaks corresponds to a particular residue, with the chemical shift of the NH backbone protons along the x-axis ( $\omega_2$ ); for maximal clarity, residues are labeled near their NH-side-chain cross-peaks. Changes in chemical shifts upon calcium addition can be clearly observed for several NH protons. Residues are labeled according to their position in the peptide.

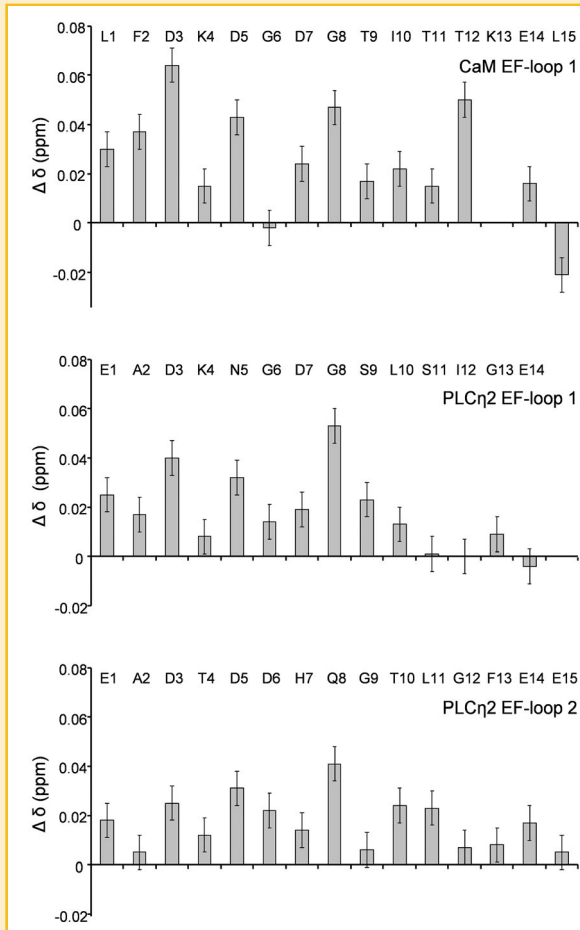


Fig. 3. Overview of the influence of  $\text{Ca}^{2+}$  (5 mM) on backbone amide resonances of EF-loop peptides corresponding to CaM EF-loop1, PLC $\eta$ 2 EF-loop1 and PLC $\eta$ 2 EF-loop 2. Residues are labeled according to their position in the peptide; for example, D3 (PLC $\eta$ 2 loop1) corresponds to D256 in the full-length protein. The first loop of calmodulin is shown as a positive control with residues directly involved in  $\text{Ca}^{2+}$ -binding in positions 3 ("X"), 5 ("Y"), 7 ("Z"), 9 ("–Y"; backbone), 11 ("–X"), and 14("–Z<sub>1</sub>" and "–Z<sub>2</sub>"). Errors are  $\pm 0.007$  ppm.

shift perturbation reporting qualitatively on the overall effect of  $\text{Ca}^{2+}$  binding on each individual residue. The residues experiencing the largest shift perturbation were those in positions 3 ("X"), 5 ("Y"), and 8 (residue after "Z") in all three peptides. One of the side-chain amide protons of N5 ("Y"; PLC $\eta$ 2 loop 1) also experienced a clear change in shift (0.026 ppm), supporting the involvement of the adjacent carbonyl oxygen in  $\text{Ca}^{2+}$ -binding. The similarity of the chemical shift perturbation patterns also suggested that in the EF-loop 2 peptide, H7 corresponds to residue "Z", and indeed, its side-chain protons were significantly affected by the presence of  $\text{Ca}^{2+}$  (H $\epsilon$ 1:  $\Delta\delta = 0.037$  ppm; also see Fig. S2 for titration data in aqueous solution), suggesting an involvement in  $\text{Ca}^{2+}$ -binding. The side-chain amide protons of Q8 experienced smaller changes in chemical shift ( $<0.02$  ppm in DMSO- $d_6$  in the presence and absence of  $\text{Ca}^{2+}$ ; titration data could not be fitted satisfactorily). Furthermore, T12

(residue after "–X") in the CaM EF-loop 1 peptide also shifted considerably; this behavior was not observed for either of the two PLC $\eta$ 2 loops. In addition, the NH resonance for K13 (CaM EF-loop 1) was not observed in either presence or absence of  $\text{Ca}^{2+}$ , whereas the corresponding residues were readily identified in the two PLC $\eta$ 2 loop peptides. These subtle differences may hint at structural variations in the C-terminal portions of the loops. It is also noteworthy that residue "–X" usually binds through a water molecule interacting with the side-chain of this residue; whilst the serine residue in loop 1 permits this, such an interaction is not possible with the glycine "–X" residue in PLC $\eta$ 2 EF-loop 2. In summary, both loops appear to have, in vitro, the ability to bind  $\text{Ca}^{2+}$  in a manner that is similar to that of canonical EF-loops for the N-terminal portion, with likely variations in the C-terminal half.

### EF-LOOP 1 IS IMPORTANT FOR PLC $\eta$ 2 ACTIVITY

The role of the EF-hand domain in activation of PLC $\eta$ 2 was investigated by site-directed mutagenesis. This was achieved by measuring the accumulation of released inositol phosphate in intact COS7 cells expressing wild-type PLC $\eta$ 2, and five EF-hand domain mutants (D256A from EF-loop 1 and D292A, H296A, Q297A, and E304A from EF-loop 2) following treatment with monensin. Monensin is a  $\text{Na}^+/\text{H}^+$  antiporter (which acts to increase cytosolic  $\text{Na}^+$  levels) and was previously found to be extremely effective in activating exogenous PLC $\eta$ 2 in these cells. This is initiated largely by an increase in cytosolic  $\text{Ca}^{2+}$  via the mitochondrial  $\text{Na}^+/\text{Ca}^{2+}$ -exchanger as this activity has been shown to be greatly inhibited by the specific inhibitor, CPG37157 [Popovics et al., 2011].

COS7 cells transiently expressing the wild-type enzyme exhibited a concentration-dependent increase in inositol phosphate accumulation (indicative of PLC activity) in response to monensin treatment relative to empty vector control (Fig. 4A,B). Inositol phosphate accumulation was typically increased by  $\sim 4$ - to 7-fold at the highest concentration used (70  $\mu\text{M}$ ). This varied a bit between assays due to differences in transfection efficiency. A small increase in PLC activity was observed in control cells but was  $<2$ -fold higher than basal level following treatment with 70  $\mu\text{M}$  monensin. This was likely due to a  $\text{Ca}^{2+}$ -induced increase in basal PLC activity. Although PLC $\eta$ 2 is not endogenously expressed in COS7 cells [Stewart et al., 2007], multiple PLCs are present in most cell types. Increased intracellular  $\text{Ca}^{2+}$  levels would likely enhance basal activity as  $\text{Ca}^{2+}$  is required for both catalysis and membrane binding. Monensin treatment did not lead to an increase in PLC activity in cells expressing the D256A mutant, suggesting that this residue is important for  $\text{Ca}^{2+}$ -dependent PLC $\eta$ 2 activity. In cells expressing the D292A mutant, inositol phosphate release was increased but was 20% less than the wild-type enzyme. Monensin treatment of cells expressing the H296A, Q297A, or E304A mutants exhibited similar increases in inositol phosphate release as those expressing the wild-type enzyme. Immunoblotting of lysates from transfected cells with anti-PLC $\eta$ 2 antibody confirmed that wild-type and mutant proteins were expressed in each case.

### $\text{Ca}^{2+}$ -SENSITIVITY OF PLC $\eta$ 2 IS CONFERRED BY EF-LOOP 1

To establish whether the EF-hand domain is responsible for  $\text{Ca}^{2+}$ -dependent activation of PLC $\eta$ 2, we examined inositol phosphate

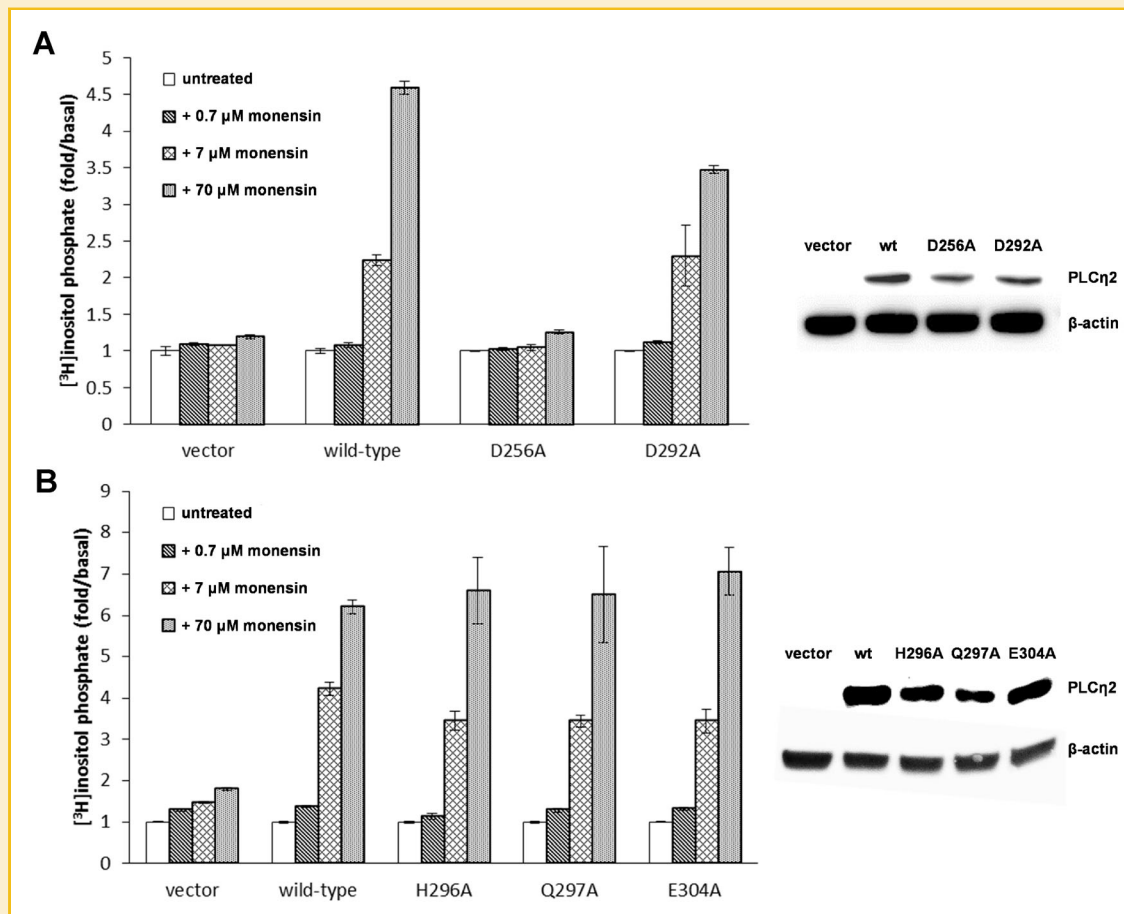


Fig. 4. Measurement of [<sup>3</sup>H]-labeled inositol phosphate accumulation in COS7 cells transfected with constructs encoding wild-type PLC $\eta$ 2 (and empty vector control) together with (A) D256A and D292A mutants and (B) H296A, Q297A, and E304A mutants in response to monensin, respectively. Results are representative of at least three experiments and expressed as  $\pm$ SEM. Western blots confirm expression of wild-type and mutant proteins in the transfected cells.

accumulation in permeabilized COS7 cells expressing the wild-type and mutant enzymes at a range of free calcium concentrations (Fig. 5). Wild-type PLC $\eta$ 2 activity was extremely sensitive to Ca<sup>2+</sup>, exhibiting a sharp increase in activity at concentrations between 0.1 and 1  $\mu$ M free Ca<sup>2+</sup> as previously reported [Popovics et al., 2011]. Endogenous PLCs (as indicated in control cells) also exhibited a Ca<sup>2+</sup>-dependent increase in activity but were less sensitive with the highest increase observed between 1 and 10  $\mu$ M free Ca<sup>2+</sup>. Significantly, 1  $\mu$ M free Ca<sup>2+</sup> was not sufficient to stimulate activity of the D256A enzyme. However, activity was observed in the presence of 10  $\mu$ M free Ca<sup>2+</sup> relative to control cells, consistent with a  $\sim$ 10-fold drop in Ca<sup>2+</sup>-sensitivity relative to the wild-type enzyme. The activity of the D292A, H296A, Q297A, and E304A enzymes in the presence of 1  $\mu$ M free Ca<sup>2+</sup> were similar to the wild-type enzyme. However, the activities of H296A, Q297A, and E304A mutants (but not the D292A mutant) were lower than that of the wild type at the highest concentrations of free Ca<sup>2+</sup> (10 mM and 100 mM; data not shown). Although the Ca<sup>2+</sup>-sensitivity of the D256A protein was 10-fold lower than the wild type enzyme this does not necessarily equate to only a 10-fold reduction in the affinity of EF-loop 1 toward Ca<sup>2+</sup> (in fact the mutation may well have a much greater effect on the

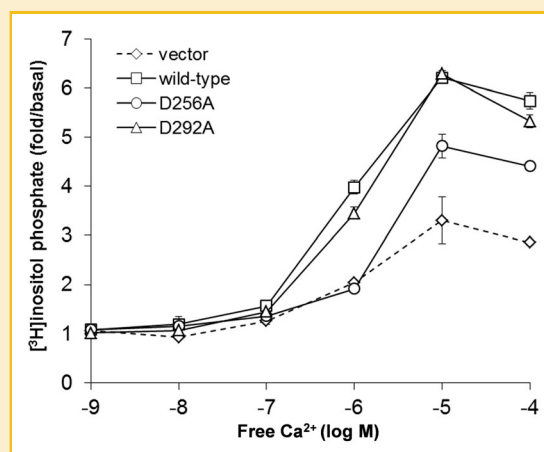
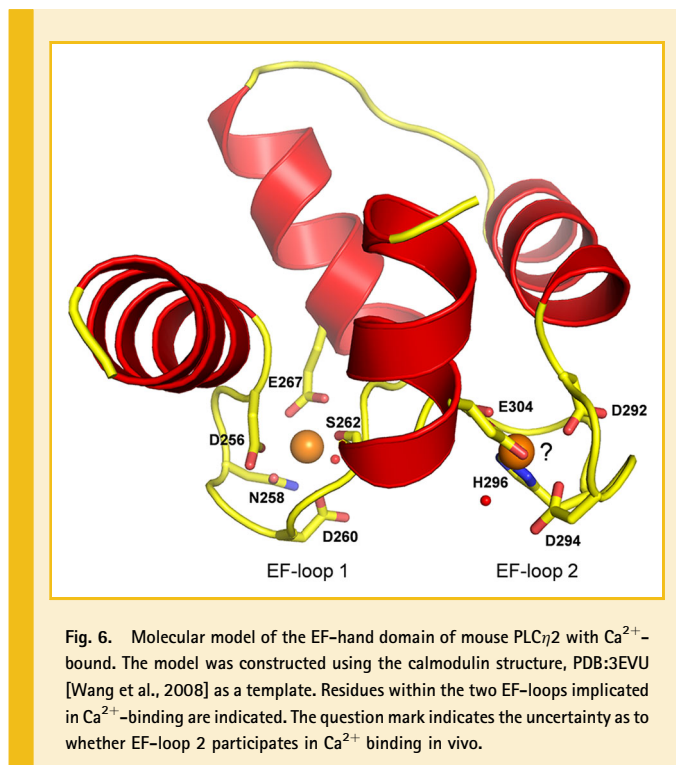


Fig. 5. Measurement of [<sup>3</sup>H]-labeled inositol phosphate accumulation in permeabilized COS7 cells transfected with constructs encoding wild-type PLC $\eta$ 2 (and empty vector control) together with D256A and D292A mutants in the presence of different concentrations of free Ca<sup>2+</sup>, respectively. Experiments were performed simultaneously. In all cases the experiments were carried out in triplicate, the results are representative of at least three experiments and are expressed as  $\pm$ SEM.

affinity). It is possible that there is another mechanism by which  $\text{Ca}^{2+}$  activates the enzyme when the free  $\text{Ca}^{2+}$  concentration is above  $1 \mu\text{M}$ . This is in part supported by the observation that  $\text{PLC}\delta 1$  exhibits a similar  $\text{Ca}^{2+}$ -sensitivity to the D256A mutant (maximal activity at  $10 \mu\text{M}$ ; Nakahara et al., 2005) yet does not possess the additional EF-hand domain found within the  $\text{PLC}\eta$  enzymes.

#### MOLECULAR MODEL OF THE $\text{PLC}\eta 2$ EF-HAND DOMAIN

In the absence of structural data for  $\text{PLC}\eta 2$ , a 3D-model of the murine  $\text{PLC}\eta 2$  EF-hand domain, consisting of two conjugated helix-loop-helix motifs, was built using the crystal structure coordinates of CaM (PDB: 3EVU; Wang et al., 2008). In the model, two 7-co-ordinate  $\text{Ca}^{2+}$  atoms are bound to each EF-loop with pentagonal bipyramidal geometries (Fig. 6). In the first EF-loop the metal binding site is composed of five residues; Asp256, Asn258, Asp260, Ser262 (backbone oxygen), and Glu267 (two ligands). A seventh O-ligand is provided by a water molecule held in position by an H-bond to the side-chain of Ser264. For the second EF-loop 2, the residues Asp292, Asp294, Thr299 (backbone oxygen), and Glu304 provide five ligands, with two coming from Glu304. In this model His296 is in a position where it seems feasible that it may also contribute to  $\text{Ca}^{2+}$  binding, in agreement with the NMR-data for the EF-loop 2 construct, but alternative models in which Gln297 provides a sidechain amide oxygen are also plausible. An additional water molecule was modeled to complete the co-ordination sphere.



## DISCUSSION

Analysis of  $\text{PLC}\eta$  sequences has revealed that this PLC class contains a novel EF-hand domain that includes a non-canonical EF-loop 2 sequence. This unusual EF-loop consists of 13 residues (rather than 12). A 13 residue EF-loop is also found within osteonectin (also called BM-40), an extracellular matrix protein where it is the first EF-loop of the pair that contains an amino acid insertion between the residues at positions 3 and 4 [Hohenester et al., 1996]. Sequence alignment of EF-hands with those from other proteins suggested the extra residue in  $\text{PLC}\eta 2$  may either have been H296 at position 5 or Q297 at position 6 of the loop.

An attempt to generate a recombinant fragment of  $\text{PLC}\eta 2$  containing the EF-hand domain for further study was unsuccessful as the resultant protein was found to be unstable (data not shown). Short EF-loop peptides have previously been used as models to probe the structural and metal-binding properties of such regions [Marsden et al., 1989; Prod'homme and Karplus, 1993]. Thereby in order to determine whether the EF-loops are able to co-ordinate  $\text{Ca}^{2+}$ , and more specifically which EF-loop residues may co-ordinate  $\text{Ca}^{2+}$ , 2D  $^1\text{H}$  TOCSY and NOESY NMR spectra were recorded for synthetic peptides corresponding to EF-loops 1 and 2 of  $\text{PLC}\eta 2$  and EF-loop 1 of CaM (as a control) in the presence and absence of  $5 \text{ mM Ca}^{2+}$ . The prevailing constitutional and conformational equilibria render a structure determination from the peptide NMR data impractical, but analysis of chemical shift perturbation of amide protons upon  $\text{Ca}^{2+}$ -binding gave qualitative hints on residues likely involved in  $\text{Ca}^{2+}$  co-ordination. The differences in amide proton shifts suggested that the N-terminal portions of both  $\text{PLC}\eta 2$  EF-loop peptides are likely to co-ordinate the metal in a similar manner to that of the canonical CaM EF-loop 1, but greater differences were seen in the C-terminal portions of the loops. Of key importance was to attempt to determine whether the side-chain from either H296 or Q297 could provide the "Z" ligand within the second EF-loop of  $\text{PLC}\eta 2$ . Interestingly, Q297 is fully conserved among vertebrate  $\text{PLC}\eta 2$  sequences (Fig. S1). Glutamine side-chains commonly provide  $\text{Ca}^{2+}$ -ligands in proteins [Harding, 2004]. However, the involvement of protein histidine side-chains in  $\text{Ca}^{2+}$ -binding has also been reported [Yoshioka et al., 1997; Eichinger et al., 1999; Bouckaert et al., 2000], although this particular interaction is quite rare [Harding, 2004]. A large shift difference recorded for the side-chain protons of H7 in the  $\text{PLC}\eta 2$  EF-loop 2 peptide, characterized by a defined dependence on  $\text{Ca}^{2+}$  concentration that can be fitted with a single dissociation constant, suggested that this residue (which corresponds to H296 in the full length protein) is a strong candidate for providing the "Z" ligand for  $\text{Ca}^{2+}$ -binding in this loop. Hence, both isolated  $\text{PLC}\eta 2$  EF-loops were capable of interacting with  $\text{Ca}^{2+}$  in vitro. A molecular model of the EF-hand domain was constructed based upon the structure of CaM with  $\text{Ca}^{2+}$  ions bound at each of the two EF-loops. Only minor rearrangements were required to accommodate the metal in each case, suggesting that both sites could potentially participate in  $\text{Ca}^{2+}$ -binding in the  $\text{PLC}\eta 2$  protein. The model supported the involvement of H296 contributing to  $\text{Ca}^{2+}$ -binding in EF-loop 2, as suggested by the peptide experiments.

Supported by the information gleaned from the NMR studies, the role of the EF-hand domain in  $\text{Ca}^{2+}$ -induced  $\text{PLC}\eta 2$  activation was

investigated by site-directed mutagenesis in live cells. The D256 residue within EF-loop 1 was mutated to alanine. This particular residue was chosen to be substituted as it is conserved within EF-loops from other proteins and is known to provide the “X” ligand. Involvement of this residue in binding  $\text{Ca}^{2+}$  is also supported by the peptide NMR data; the amide protons of the corresponding residue in the EF-loop 1 peptide undergo a characteristic chemical shift change upon addition of  $\text{Ca}^{2+}$ . Four residues were mutated to alanine within EF-loop 2. These included D292 and E304 which were chosen because residues at these positions are known to provide  $\text{Ca}^{2+}$ -binding “X” and “-Z” ligands, respectively in (canonical) EF-loops of other proteins [Gifford et al., 2007]. The H296 and Q297 residues were also mutated as likely candidates to constitute the “extra residue” uniquely found within PLC $\eta$ 2 EF-loop 2 sequences, and either could correspond to the “Z” residue.

As previously reported, cells expressing wild-type PLC $\eta$ 2 exhibited a concentration-dependent increase in inositol phosphate accumulation in response to monensin treatment [Popovics et al., 2011]. The D256A mutation had a dramatic effect upon the ability of the enzyme to respond to monensin-induced elevations in  $[\text{Ca}^{2+}]_i$ . In contrast, increases in inositol phosphate accumulation were observed in cells expressing the D292A, H296A, Q297A, and E304A mutants that were similar to those observed in cells expressing the wild-type enzyme. It should be noted that this effect was slightly reduced (ca. 20%) in D292A. If  $\text{Ca}^{2+}$ -binding to EF-loop 2 was important for PLC $\eta$ 2 activity then it would be expected that at least one of the four mutations would influence  $\text{Ca}^{2+}$ -binding and greatly perturb the ability of the enzyme to respond to monensin-induced  $\text{Ca}^{2+}$  influx. These results suggest that  $\text{Ca}^{2+}$ -binding to EF-loop 1, but not EF-loop 2 is important for PLC $\eta$ 2 activity.

To determine whether the EF-hand domain is directly involved in sensing  $\text{Ca}^{2+}$ , inositol phosphate accumulation was examined in permeabilized COS7 cells transiently expressing the wild-type and mutant proteins at a range of free  $\text{Ca}^{2+}$  concentrations. The wild-type enzyme exhibited the greatest increase in activity at free  $\text{Ca}^{2+}$  concentrations between 0.1  $\mu\text{M}$  and 1  $\mu\text{M}$  relative to the control cells. This finding is in agreement both with our previous observations [Popovics et al., 2011], and studies by Nakahara et al. (2005) who have measured the activity of recombinant PLC $\eta$ 2 in the presence of  $\text{Ca}^{2+}$ . As expected the D256A mutant was unable to respond to 1  $\mu\text{M}$  free  $\text{Ca}^{2+}$  but was active in the presence of 10  $\mu\text{M}$  free  $\text{Ca}^{2+}$ . This corresponds to a  $\sim$ 10-fold reduction in  $\text{Ca}^{2+}$  sensitivity. Basal PLC activity due to endogenously expressed PLC enzymes was also optimal at 10  $\mu\text{M}$  free  $\text{Ca}^{2+}$  and was similar to that previously observed for PLC $\delta$ 1 [Nakahara et al., 2005]. Hence when  $\text{Ca}^{2+}$ -binding to EF-loop 1 is disrupted, PLC $\eta$ 2 exhibits a  $\text{Ca}^{2+}$ -dependence similar to that of PLC $\delta$ 1. The EF-loop 2 mutations had no effect upon  $\text{Ca}^{2+}$ -sensitivity, as with the wild-type enzyme, these mutants exhibited maximal activity in the presence of 1  $\mu\text{M}$  free  $\text{Ca}^{2+}$  (data not shown). Interestingly, the activity of the D292A mutant at 1  $\mu\text{M}$  of free  $\text{Ca}^{2+}$  was  $\sim$ 20% less than wild-type PLC $\eta$ 2 (as observed in the intact cell assay); this may be due to a difference in protein expression or the mutation may cause a conformational change that alters the ability of the EF-hand to “communicate” with the catalytic domains. The permeabilized cell experiments largely reflected the results

observed in the intact cells treated with monensin given that this agent is likely to increase  $[\text{Ca}^{2+}]_i$  to  $\sim$ 1  $\mu\text{M}$ .

Collectively, these results suggest that EF-loop 1 of PLC $\eta$ 2 is able to bind submicromolar concentrations of free  $\text{Ca}^{2+}$ , which in turn alter the conformation of the enzyme such that its ability to hydrolyze  $\text{PIP}_2$  is enhanced. The EF-loop 2 mutations had no great effect upon activity suggesting this loop may either not bind  $\text{Ca}^{2+}$  at physiological concentrations, or that  $\text{Ca}^{2+}$ -binding does not affect PLC $\eta$ 2 activity. Although the synthetic EF-loop 2 peptide was shown to bind to  $\text{Ca}^{2+}$  in a similar manner to either EF-loop 1 or a CaM control peptide (albeit at higher than physiological concentration), this loop may exhibit similar behavior when part of the full length protein. An EF-hand domain with a non-functional second EF-loop is not without precedent; the sarcoplasmic  $\text{Ca}^{2+}$ -binding protein (SCP) from the sandworm, *Nereis diversicolor* possesses an EF-hand domain with a non-functional second loop [Vijay-Kumar and Cook, 1992]. Although this protein is thought to function as a  $\text{Ca}^{2+}$  buffer rather than an effector protein, NMR and circular dichroic studies of apo- and metal bound-SCP reveal that the binding of 1 mole equivalent of  $\text{Ca}^{2+}$  invokes a significant change in the conformation of the protein; SCP has a highly unstructured apo state which is switched to the native state upon binding of the first  $\text{Ca}^{2+}$  ion [Prêcheur et al., 1996]. It is therefore conceivable that  $\text{Ca}^{2+}$ -binding at the first EF-loop of PLC $\eta$ 2 alone is sufficient to allosterically control catalysis. The function of the EF-hand-like domain (residues 317–399) in PLC $\eta$ 2 is unknown but it is possible that it plays a role in translating  $\text{Ca}^{2+}$ -binding by the EF-hand domain discussed here to the catalytic domain.

In conclusion, we provide the first evidence that the EF-hand domain of PLC $\eta$ 2 is responsible for activation by  $\text{Ca}^{2+}$  and highlight the involvement of EF-loop 1 in mediating this effect. Further work is required to establish whether both EF-loops bind  $\text{Ca}^{2+}$  under physiological conditions, to resolve whether EF-loop 2 binds  $\text{Ca}^{2+}$  too weakly, or with in principle sufficient strength, but without influencing enzyme activity.

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