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Drayer, A. Lyndsay; Meima, Marcel E.; Derks, Mari W.M.; Tuik, Ronald; Haastert, Peter J.M. van

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Mutation of an EF-hand Ca^{2+}-binding motif in phospholipase C of *Dictyostelium discoideum*: inhibition of activity but no effect on Ca^{2+}-dependence

A. Lyndsay DRAYER, Marcel E. MEIMA, Mari W. M. DERKS, Ronald TUIK and Peter J. M. VAN HAASTERT*

Department of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

Phosphoinositide-specific phospholipase C (PLC) is dependent on Ca^{2+} ions for substrate hydrolysis. The role of an EF-hand Ca^{2+}-binding motif in Ca^{2+}-dependent PLC activity was investigated by site-directed mutagenesis of the *Dictyostelium discoideum* PLC enzyme. Amino acid residues with oxygen-containing side chains at co-ordinates x, y, z, $-x$ and $-z$ of the putative Ca^{2+}-binding-loop sequence were replaced by isoleucine (x), valine (y) or alanine (z, $-x$ and $-z$). The mutated proteins were expressed in a *Dictyostelium* cell line with a disrupted *plc* gene displaying no endogenous PLC activity, and PLC activity was measured in cell lysates at different Ca^{2+} concentrations. Replacement of aspartate at position x, which is considered to play an essential role in Ca^{2+} binding, had little effect on Ca^{2+} affinity and maximal enzyme activity. A mutant with substitutions at both aspartate residues in position x and y also showed no decrease in Ca^{2+} affinity, whereas the maximal PLC activity was reduced by 60%. Introduction of additional mutations in the EF-hand revealed that the Ca^{2+} concentration giving half-maximal activity was unaltered, but PLC activity levels at saturating Ca^{2+} concentrations were markedly decreased. The results demonstrate that, although the EF-hand domain is required for enzyme activity, it is not the site that regulates the Ca^{2+}-dependence of the PLC reaction.

INTRODUCTION

The enzyme phosphoinositide-specific phospholipase C (PLC) generates Ins(1,4,5)P_3 and diacylglycerol by hydrolysis of PtdIns(4,5)P_2. Ins(1,4,5)P_3 plays an important role in Ca^{2+} mobilization by releasing Ca^{2+} from internal stores [1,2], and diacylglycerol is the physiological activator of protein kinase C [3]. Besides its function as a producer of these classical second messengers, PLC may play a role in remodelling of the cytoskeleton through regulation of the PtdIns(4,5)P_2 interaction with actin-binding proteins such as profilin and gelsolin [4,5].

On the basis of sequence conservation, the mammalian PLC isoforms are classified into three distinct families, β, γ and δ, which are regulated by separate mechanisms. Members of the PLC-β family are activated by G-protein-linked receptors [6,7], and PLC-γ isoforms are activated by tyrosine kinase-linked receptors (see ref. [8]). The mechanisms involved in PLC-δ activation have not been resolved so far. A RhoGAP protein has been identified which associates with and activates PLC-δ_1, suggesting a mechanism in which the small G-protein Rho is involved in PLC-δ regulation [9].

Lower eukaryotes provide systems in which to study the function of PLC by analysing mutants. In *Drosophila* the *norpA* gene, which encodes a protein similar to bovine retinal PLC-β, appears to be involved in phototransduction [10]. The micro-organisms *Saccharomyces cerevisiae* and *Dictyostelium discoideum* contain a PLC-δ-like gene [11,12]. In *S. cerevisiae* deletion of the PLC gene resulted in retarded cell growth [13]. Deletion of the PLC gene in the slime mould *D. discoideum* resulted in cells containing no detectable PLC activity, yet growth and development were unaffected [14]. Ins(1,4,5)P_3 levels were only slightly lower in the *plc*$^-$ mutant compared with wild-type cells, which suggests that there are alternative pathways for generating Ins(1,4,5)P_3 besides PLC [15].

Structural requirements of PLC-β, -γ and δ for enzyme activity have been identified using bacterial and mammalian expression systems. The domains A and B, containing conserved amino acids found in all PLC isoforms, are essential for enzyme activity and are thought to form the catalytic core [16,17]. In PLC-γ the region between the conserved A and B domains contains the *src* homology (SH) domains SH2 involved in the PLC–tyrosine kinase interaction, and an SH3 domain which targets the enzyme to cytoskeletal components [18]. In PLC-β the N-terminal domain is required for activation by G-protein $\beta\gamma$-subunits, and the large C-terminal domain after the conserved B domain is required for activation by α-subunits [19–21]. The N-terminal domain in PLC-δ, containing a pleckstrin homology (PH) domain, has been shown to form a high-affinity binding site for PtdIns(4,5)P_2 [22].

Ca^{2+} is an important regulator of PLC, yet little is known about the mechanism by which Ca^{2+} stimulates PLC activity. The sequences of several PLC isoforms predict an EF-hand motif, a domain found in many Ca^{2+}-modulated proteins [23,24]. The EF-hand motif consists of 29 amino acids arranged in a helix–loop–helix conformation, with Ca^{2+} binding in the loop region [25]. In this study we investigated the role of the putative Ca^{2+}-binding domain for Ca^{2+}-dependent PLC activity, by introducing point mutations into the EF-hand of *Dictyostelium* PLC. The altered proteins were expressed in a *Dictyostelium* mutant strain with a disrupted *plc* gene containing no endogenous PLC activity. The results show that there is no difference in Ca^{2+}-dependence between PLC with a complete EF-hand and mutated PLC proteins, but that the maximal enzyme activity is affected by the mutations.

EXPERIMENTAL

Generation of *plc*$^-$ cells

A vector was constructed for disruption of the endogenous DdPLC gene by homologous recombination using *ura*$^-$

Abbreviation used: PLC, phosphoinositide-specific phospholipase C.

* To whom correspondence should be addressed.

complementation. A 3.7 kb *Cla*I fragment from pDU3B1 [26] encoding the *Dictyostelium* UMP synthase gene was ligated between two internal *Cla*I sites in a DdPLC cDNA construct to create the plasmid pUraPLCko (see Figure 1a). As in the previously reported G418-resistant *plc*$^-$ cell line HD10, a selection marker for a double-crossover event by homologous recombination, the tRNA$^{Glu(UAA)}$ suppressor gene, was included with no adverse effects [14].

A uracil auxotroph *D. discoideum* strain DH1 (a gift from P. N. Devreotes, The Johns Hopkins University, Baltimore, MD, U.S.A.) was grown axenically in minimal (FM) medium [27] supplemented with uracil (100 μg/ml). DH1 cells were transformed with pUraPLCko by electroporation [28] and grown in minimal medium (without added uracil) until colonies appeared. Transformants were clonally selected on agar plates with *Klebsiella aerogenes*, and then the clones were grown in minimal medium. Southern blotting on genomic DNA from DH1 and transformants using probes specific for the conserved A and B domains of DdPLC was performed as described [14]. One *plc*$^-$ clone, named DH1.19, was selected and used for further experiments.

Point mutation of the EF-hand

A cDNA fragment consisting of the coding region and 3′ untranslated region of DdPLC (described in ref. [14]) was incorporated into the bacterial expression plasmid pBluescript SK(−) (Stratagene) to yield pPLC-blue. Mutation of DdPLC at amino acid position 490, and positions 490+492 (in positions x, or $x+y$ of the Ca^{2+}-binding-loop sequence, see Figure 3b), were performed by site-directed mutagenesis using pPLC-blue as template [29]. The primer used for mutation of the EF-hand had the sequence 5′-TCAAACTGATATCAATG(A/T)TGATGATGGT-3′ (bases 1746–1773 of DdPLC). The altered nucleotides are in bold. Clones carrying mutations could be detected by screening for the presence of an additional *Eco*RV restriction site in DdPLC. Clones encoding one or two mutations in the EF-hand were selected by sequence analysis [30]. Construction of a plasmid for expression of full-length DdPLC cDNA in *Dictyostelium* using the BS18 vector, pPLC-BS18, has been described previously [12]. A 1.4 kb *Nco*I–*Hin*dIII fragment of DdPLC cDNA containing the mutations in pPLC-blue was isolated and inserted into *Nco*I–*Hin*dIII-digested pPLC-BS18. The mutated DdPLC sequences cloned into BS18 were named pEF1 with one mutation in the EF-hand, and pEF2 with two mutations in the EF-hand.

Mutations of DdPLC at amino acid positions 490+492+501 and 490+492+494+498+501 (in positions x, y, $-z$ or x, y, z, $-x$, $-z$ of the Ca^{2+}-binding-loop sequence, see Figure 3b) were performed by PCR [31] using pEF2 as template. Primers used were: PLC5′D, 5′-GTTCATTGTTCAGTACC-3′ (bases 1537–1553 of DdPLC); PLC5′EF, 5′-GATGCTGGTGCTGTTG(A/C)TTTAACAGCATATGATGAA-3′ (bases 1765–1800 of DdPLC); PLC3′EF, 5′-ATCATATGCTGTTAAT(T/G)CAACAGCACCAGCATCAACATT-3′ (bases 1759–1799 of non-coding DdPLC); and TN5BS3′, 5′-ACTTGATTC TTCATCGG-3′ (non-coding strand terminator sequence of BS18 vector). The position of the primers is indicated schematically in Figure 3(b). In the first step PCR was performed with primers PLC5′EF and TN5BS3′. The amplified fragment of 1 kb was digested with *Nde*I–*Hin*dIII and inserted into *Nde*I–*Hin*dIII-digested pEF2, yielding construct pEFmin, which lacks an internal DdPLC fragment of 200 bp. A second PCR was performed with primers PLC5′D and PLC3′EF resulting in a 200 bp product which was digested with *Nde*I and ligated into compatible pUC21 vector. Sequence analysis revealed a product with five mutations in the EF-hand region, as expected, and an extra product with three mutations (at positions x, y, $-z$; the $+z$ position was not mutated). The 200 bp products carrying three or five mutations were ligated into the *Nde*I site of pEFmin, and the orientation of the inserts was determined by DNA sequencing. Inserts generated by PCR were sequenced completely. The mutated DdPLC sequences cloned into BS18 were named pEF3 carrying three mutations in the EF-hand and pEF5 carrying five mutations in the EF-hand.

The *ura*$^+$ *plc*$^-$ mutant DH1.19 was transformed with plasmids pPLC-BS18, pEF1, pEF2, pEF3 and pEF5. Transformants were selected and cloned in HL5 medium [32] containing G418 at 10 μg/ml. The PLC proteins generated by cells expressing constructs with one, two, three or five mutations in the EF-hand of DdPLC were designated DdPLC-1(x), DdPLC-2(x,y), DdPLC-3(x,y,$-z$) and DdPLC-5 (x,y,z,$-x$,$-z$) respectively.

PLC assay

To prepare cell suspensions for PLC assays, exponentially growing cells were harvested by centrifugation at 300 *g* and resuspended at a density of 5×10^7 cells/ml in 40 mM Hepes/NaOH buffer, pH 6.5. Samples of cells were lysed by rapid elution through Nuclepore polycarbonate filters (pore size 3 μm). PLC activity in lysates was measured as described previously [33], with at least two independently derived clones for each mutated DdPLC construct. The Ca^{2+}-dependence of PLC activity was assayed in the presence of Ca^{2+}/EGTA buffers, containing different concentrations of added $CaCl_2$ stock solutions. EGTA was added to the cells before lysis, and the final concentration of EGTA was fixed at 5.36 mM for all experiments. Free Ca^{2+} concentrations were calculated as described by Barfati [34], solely taking the added EGTA and $CaCl_2$ solutions into account. As the reactions were performed in crude cell lysates, it should be noted that components interfering with the Ca^{2+}/EGTA buffer could be present. However, as all experiments were performed under the same conditions, the effects observed in this study would not depend on the difference between calculated free Ca^{2+} concentration and the actual final concentration of free Ca^{2+} in the reaction.

The data were fitted to the equation:

$$\text{PLC activity} = \text{PLC}_{\text{max.}}/\{1+([Ca^{2+}]/[Ca^{2+}]_{50})^{-h}\}$$

where $\text{PLC}_{\text{max.}}$ is the maximal PLC activity (in pmol of Ins[1,4,5]P_3 produced/min per μg), $[Ca^{2+}]_{50}$ is the Ca^{2+} concentration at which PLC activity is half-maximal, and h is the Hill coefficient.

Western-blot analysis

Cells were resuspended in 40 mM Hepes buffer, as described above for the PLC assay, and protein-separation sample buffer was added [35]. Samples for Western-blot analysis contained 40 μg of protein from total cell lysates for DH1 or the mutant preparations and 5 μg of protein from total cell lysates for analysis of the DdPLC EF-hand mutants. Samples were boiled before separation by SDS/PAGE [36], and transferred to nitrocellulose [37]. Blots were incubated with antiserum raised against recombinant DdPLC [14] and developed using the ECL detection kit (Amersham). Protein concentration was determined by a Bio-Rad protein assay with BSA as standard. G-protein β-subunit-specific antiserum [38] was used as an internal control to compare protein levels in the lanes of a blot.

RESULTS

Generation of *plc*⁻ cells and rescue of Ca^{2+}-dependent PLC activity

We have recently described a *Dictyostelium* mutant HD10, in which the endogenous PLC gene DdPLC had been disrupted [14]. As mutant HD10 was obtained by a transformation procedure using G418 selection, another *plc*⁻ mutant was required for the present study to express altered proteins from the *Dictyostelium* expression vector BS18 (which confers G418-resistance).

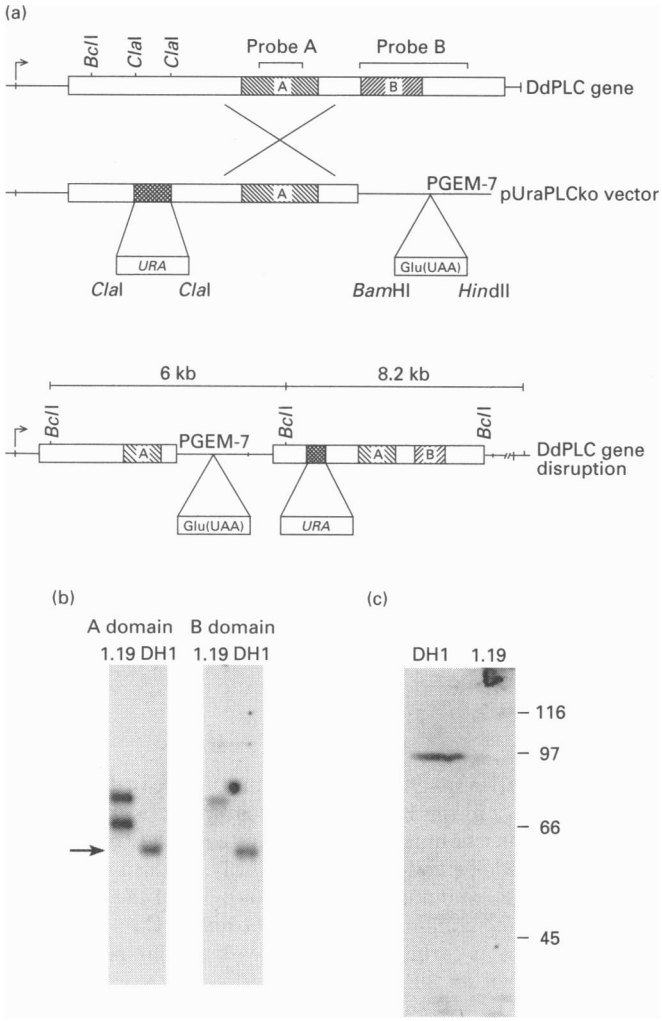

Figure 1 Disruption of the DdPLC locus by gene targeting

(**a**) In the construct pUraPLCko the internal *Cla*I fragment of a cDNA clone, containing part of the DdPLC sequence up to the conserved B domain, is replaced with the *Dictyostelium* UMP synthase gene (*URA*). (**b**) Southern-blot analysis of DdPLC in strain DH1 and cells that had been transformed with pUraPLCko was performed on genomic DNA digested with *Bcl*I. The blot was hybridized with a probe consisting of the conserved A domain of PLC (left), stripped and then probed with a B-domain probe of DdPLC. In the parental strain DH1, the probes detect a ~ 4.5 kb fragment (indicated by an arrow) which is absent from the mutant HD1.19 (labelled as 1.19). Two copies of the region encoding the A domain are present in HD1.19, and one copy of the B domain. The results are consistent with a single-crossover event between the pUraPLCko construct and the DdPLC locus, as indicated in (**a**). The $tRNA^{Glu(UAA)}$ suppressor gene was included in the disruption construct to select for a double-crossover event, but was not effective as described previously [14]. (**c**) Immunoblot of proteins from DH1 and mutant HD1.19 with antiserum specific for DdPLC. In DH1, a protein of ~ 97 kDa is detected which approximates the calculated size of 92 kDa for DdPLC; in HD1.19 no PLC protein is detected. Numbers on the right indicate the migration position of molecular-mass standards in kDa.

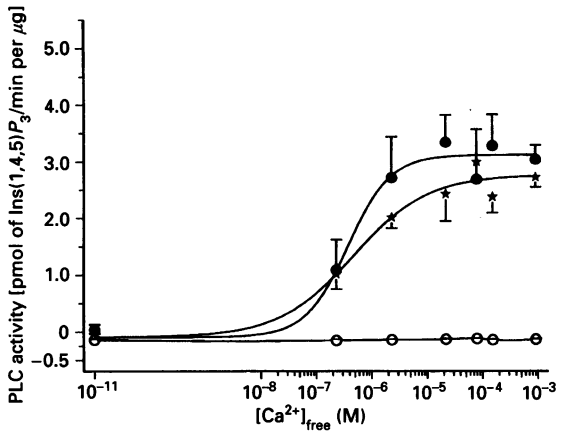

Figure 2 Ca^{2+}-dependence of DdPLC

Vegetatively growing HD1.19 *plc*⁻ cells (○) and HD1.19 cells transformed with the BS18 vector containing the complete DdPLC cDNA (●, ★; these represent two independent transformants expressing DdPLC) were lysed in the presence of EGTA. PLC activity [pmol of Ins(1,4,5)P_3 produced/min per μg of total protein] was assayed in the crude lysates using endogenous PtdIns(4,5)P_2 as substrate. The concentration of added free Ca^{2+} was varied as indicated. Data presented are from a single experiment performed in triplicate. Standard deviations are shown when larger than symbol size.

A G418-sensitive *plc*⁻ mutant was isolated by transforming the *ura*⁻ strain DH1 with the plasmid pUraPLCko described in Figure 1(a). Transformants were selected for *ura*⁻ complementation by growth in minimal medium. A number of independent clones was obtained, some of which had integrated the vector into the DdPLC locus as observed by Southern-blot analysis. The results for one clone which was used in further experiments, HD1.19, are presented in Figure 1. The Southern blot of genomic DNA demonstrated that a single crossover event had occurred in the DdPLC locus of HD1.19 cells. Disruption of the DdPLC gene resulted in a mutant containing no PLC protein when analysed by immunoblot using DdPLC-specific antiserum (Figure 1c), and no detectable PLC activity (see Figure 2). HD1.19 *plc*⁻ cells showed a normal growth and developmental pattern compared with control cells, as was previously observed for HD10 *plc*⁻ cells.

HD1.19 cells were transformed with plasmid BS18 containing the complete DdPLC cDNA sequence downstream from the actin-15 promoter. Figure 2 shows the PLC activity measured in cell lysates at different concentrations of free Ca^{2+}. Expression of DdPLC in HD1.19 cells restored PLC activity to the *plc*⁻ mutant. The PLC activity was dependent on the presence of Ca^{2+}, showing no activity in the absence of added Ca^{2+} and reaching a maximum at approx. 5 μM free Ca^{2+}. Studies on endogenous PLC activity in wild-type *Dictyostelium* lysates showed a dose–response curve for Ca^{2+} that was bell-shaped with a maximal activity of 0.1 pmol of Ins(1,4,5)P_3 produced/min per μg of total protein [33]. In contrast, in cells expressing DdPLC from the strong actin-15 promoter, a maximum PLC activity level of 3 pmol of Ins(1,4,5)P_3 produced/min per μg was reached which was sustained at high Ca^{2+} concentrations.

Mutation of the EF-hand

A search of the SWISS-PROT database revealed that mammalian PLC-γ1, PLC-δ1, *S. cerevisiae* PLC and *D. discoideum* PLC contain an EF-hand motif detected by the PROSITE EF-hand pattern [39]. In PLC-γ1, PLC-δ1 (containing two putative EF-

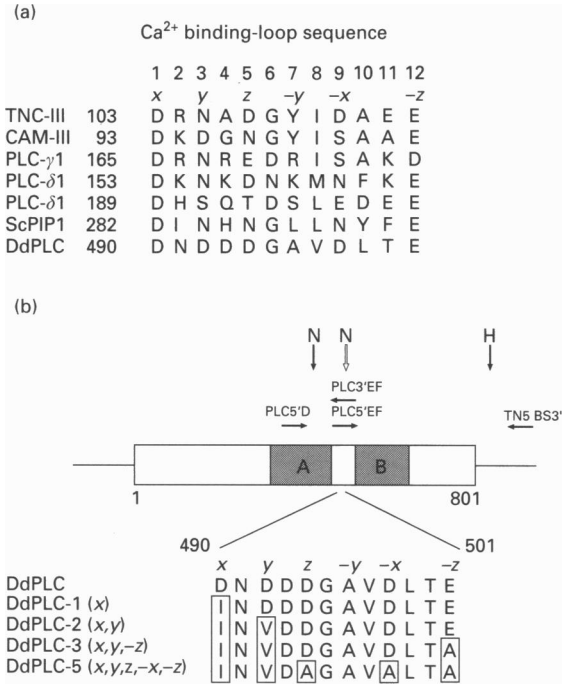

Figure 3 (a) Alignment of the Ca^{2+}-binding loop sequences of EF-hand III of rabbit skeletal-muscle troponin C (TnC-III) and bovine brain calmodulin (CaM-III), and the predicted EF-hands of rat PLC-γ1, PLC-δ1, *S. cerevisiae* PLC (ScPIP1) and *D. discoideum* PLC (DdPLC) and (b) point mutation of the EF-hand of DdPLC

(**a**) The numbers indicate the position of the amino acid residues within the protein. The sequence positions in the Ca^{2+}-binding-loop domain are numbered 1–12, starting with the N-terminal residue. Ca^{2+} is co-ordinated directly by oxygen atoms provided by the side chains of residues at positions 1(x), 3(y), 5(z) and 12($-z$). The side chain of the residue at position 9($-x$) ligates Ca^{2+}either directly or indirectly via a water molecule. The residue at position 7($-y$) participates in Ca^{2+} ligation via its backbone carbonyl oxygen. The established nomenclature of an octahedral arrangement of the ligands is used, although the residue at $-z$ (usually a glutamate) has been shown to use both its side-chain oxygens, resulting in seven oxygen ligands and a pentagonal bipyramidal Ca^{2+} co-ordination [44]. (**b**) The EF-hand in *Dictyostelium* PLC is located in the region between the conserved A and B domains. The amino acids that were mutated are boxed. The positions of the primers used in PCR are schematically indicated by horizontal arrows at the corresponding regions in the Figure. The positions of the restriction sites *Nco*I (N) and *Hin*dIII (H) are indicated by vertical arrows (the open arrow indicates the *Nde*I site generated by PCR, see the Experimental section).

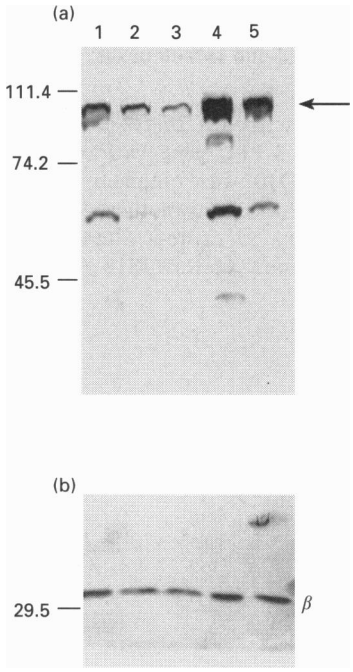

Figure 4 Expression of normal and mutated DdPLC in *Dictyostelium*

Protein samples from *plc*$^-$ cells expressing DdPLC (lane 1), DdPLC-1(x) (lane 2), DdPLC-2(x,y) (lane 3), DdPLC-3($x,y,-z$) (lane 4) and DdPLC-5($x,y,z,-x,-z$) (lane 5) were analysed by Western blot with DdPLC-specific antiserum (**a**). The blot was stripped and incubated with antiserum against the G-protein β-subunit, which is expressed constitutively in *Dictyostelium* cells [38], to compare the amount of protein loaded in each lane (**b**). Lanes 2 and 3 showed less intense staining with the β-subunit antiserum than lanes 1, 4 and 5, indicating that a lower amount of total protein was loaded in these lanes. Numbers on the left indicate migration positions of molecular-mass standards in kDa. The arrow shows the position of full-length PLC protein of approx. 97 kDa. In addition, samples contained a number of smaller products which probably represent cleaved PLC protein. The degradation products were detected when high levels of PLC proteins were analysed, explaining the higher amounts of smaller proteins in lanes 1,4 and 5 than in lanes 2 and 3.

hands) and *S. cerevisiae* PLC, the EF-hand domains are located in the N-terminal region of PLC. In *Dictyostelium* PLC the EF-hand is located between the conserved A and B domains. Figure 3(a) compares the amino acid sequences in Ca^{2+}-binding loops of troponin C and calmodulin with the putative binding loops in EF-hands of PLC isoforms. Binding of Ca^{2+} occurs within the 12-residue loop region and involves six residues at positions 1 ($+x$), 3 ($+y$), 5 ($+z$), 7 ($-y$), 9 ($-x$) and 12 ($-z$) [23]. The carboxyl, amide and hydroxyl side chains of amino acid residues at positions x, y, z, $-x$ and $-z$ in the loop sequence play an important role in Ca^{2+}-binding by co-ordinating the ion directly with the oxygen-containing side chains. The oxygen at position $-y$ comes from the backbone and can be supplied by any amino acid.

Potential Ca^{2+}-binding sites in the EF-hand of DdPLC were replaced by amino acids with aliphatic side chains as outlined in Figure 3(b), while keeping the rest of the DdPLC protein intact. One of the most conserved amino acids in the Ca^{2+}-binding loop from a variety of EF-hand proteins is an aspartate residue, corresponding to Asp-490 in DdPLC. In a first construct Asp-490 at position x in the EF-hand was changed to isoleucine. In a second construct we mutated both asparate residues at positions x and y to isoleucine and valine respectively. A third construct, carrying an additional mutation at position $-z$ of the loop, was made by replacing glutamate, which co-ordinates Ca^{2+} with both oxygen atoms of its carboxylate group, with an alanine. Finally, in a fourth construct all potential co-ordinating side chains at positions x, y, z, $-x$ and $-z$ in the Ca^{2+}-binding loop were mutated.

The DdPLC cDNA constructs carrying mutations in the EF-hand were cloned into the expression vector BS18 and transformed to HD1.19 *plc*$^-$ cells. Figure 4 shows an immunoblot analysis of *Dictyostelium* lysates from HD1.19 cells expressing unmutated (lane 1) and mutated DdPLC proteins (lanes 2–5). The level of expression of PLC protein differed between the cell lines. As can be seen from Figure 4, cell lines expressing DdPLC with three or five mutations in the EF-hand contained higher levels of PLC protein than the sample expressing unmutated DdPLC, whereas cell lines expressing DdPLC with one or two mutations in the EF-hand contained lower levels of PLC protein. The lower levels of PLC protein observed in lanes 2 and 3 could in part be explained by reduced total protein levels, as determined with the G-protein β-subunit which was used as an internal control (Figure 4b).

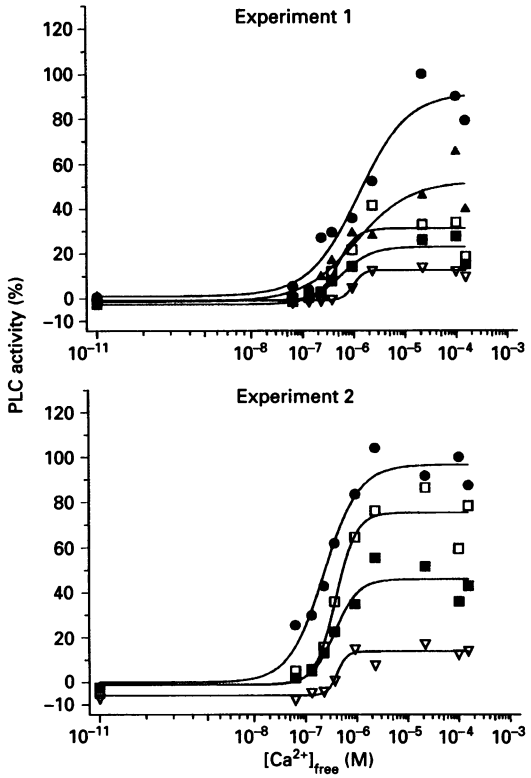

Figure 5 Effect of EF-hand mutations on PLC activity

Dictyostelium plc⁻ cells were transformed with the expression vector BS18 containing unmutated and mutated DdPLC cDNA constructs. Data are expressed as percentage of the enzyme activity present in unmutated DdPLC at 0.1 mM free Ca^{2+}. Each point represents triplicate determinations from a single experiment. The curve was obtained by fitting the data to a modified form of the Hill equation (see the Experimental section). ●, DdPLC; ▲, DdPLC-1(*x*); □, DdPLC-2(*x*,*y*); ■, DdPLC-3(*x*,*y*,−*z*); ▽, DdPLC-5(*x*,*y*,*z*,−*x*,−*z*).

Table 1 Kinetic properties of normal and mutated *Dictyostelium* PLC

The data from Figure 5 and similar experiments were used to derive the pCa_{50} (pCa at which PLC activity is half-maximal), the $PLC_{max.}$ (maximal PLC activity) and the Hill coefficient (*h*) using the equation:

$$\text{PLC activity} = PLC_{max.}/\{1+([Ca^{2+}]/[Ca^{2+}]_{50})^{-h}\}$$

Data are presented as means ± S.D. *n* indicates the number of independent experiments.

	n	pCa_{50}	*h*	$PLC_{max.}$ (pmol/min per μg)
DdPLC	4	6.1 ± 0.3	1.0 ± 0.2	4.0 ± 1.7
DdPLC-1(*x*)	3	6.1 ± 0.3	1.5 ± 0.8	3.0 ± 1.1
DdPLC-2(*x*,*y*)	6	6.3 ± 0.4	1,3 ± 0.7	1.7 ± 0.8
DdPLC-3(*x*,*y*,−*z*)	6	6.3 ± 0.1	2.0 ± 0.4	0.6 ± 0.5
DdPLC-5(*x*,*y*,*z*,−*x*,−*z*)	5	6.3 ± 0.1	2.8 ± 1.2	0.3 ± 0.2

Effect of EF-hand mutations on PLC activity

The concentration of free Ca^{2+} required to activate PLC in lysates from cells expressing unmutated DdPLC was compared with that in cells expressing EF-hand-mutated DdPLCs. The results of two representative experiments are shown in Figure 5, and in Table 1 the results of a number of experiments are summarized. The Ca^{2+}-activation experiments revealed that mutation of the EF-hand did not result in an altered affinity of PLC for Ca^{2+}. Half-maximal PLC activity was observed at pCa values between 6.1 and 6.3. All PLC proteins reached their maximal activity at 5 μM free Ca^{2+}; the rate of hydrolysis did not increase with higher concentrations of Ca^{2+}. The Hill coefficient of unmutated DdPLC was approximately 1, but increased in the mutated proteins (see Table 1). This suggests that the EF-hand-mutated DdPLC proteins are activated by a positively co-operative process.

A striking difference was seen in the rate of PtdIns(4,5)P_2 hydrolysis between DdPLC and mutated DdPLCs. The average ratios of $PLC_{max.}$ for DdPLC containing one, two, three or five mutations in the EF-hand relative to unmutated DdPLC were respectively 0.75, 0.4, 0.15 and 0.08. These values were obtained by comparing enzyme activities in total cell lysates, suggesting that a difference in maximal PLC activity might be due to a difference in expression level of the PLC protein in the cells. For DdPLC proteins with one or two mutations in the EF-hand, the lower $PLC_{max.}$ levels could partly be attributed to lower expression of the PLC protein. However, this is not the case for DdPLC with three or five mutations. Figure 4 shows an immunoblot analysis of the same cell preparations as were used for measuring PLC activities in Experiment 1 of Figure 5. The DdPLC proteins with three or five mutations in the EF-hand were expressed at higher levels than unmutated DdPLC, yet the rate of hydrolysis was decreased by 80–90%. The observed decrease in maximal PLC activity is therefore not due to a decrease in expression of the PLC proteins in the cells, but is caused by the mutations in the DdPLC protein. We conclude that increasing the number of mutations in the EF-hand of DdPLC results in a progressive decrease in maximal PLC activity, without affecting the affinity of the enzyme for Ca^{2+}.

DISCUSSION

PtdIns(4,5)P_2 hydrolysis catalysed by PLC is highly dependent on the presence of Ca^{2+} (for examples see refs.[22,33,40]). Ca^{2+} could regulate PLC activity by binding directly to a Ca^{2+}-binding site in the PLC protein, by binding to an intermediate protein that regulates PLC activity, or by interacting with the substrate. Two different Ca^{2+}-binding sites have been predicted in PLC proteins. One has been proposed in mammalian PLC isoforms in a region with some homology to the Ca^{2+}-dependent phospholipid-binding domain of cytosolic phospholipase A_2 and protein kinase C [41]. The Ca^{2+}-dependent phospholipid-binding domain of cytosolic phospholipase A_2 has been demonstrated to be involved in Ca^{2+}-dependent translocation of the enzyme to the membrane. *Dictyostelium* PLC shows a similar homology to the consensus sequence of the Ca^{2+}-dependent phospholipid-binding domain at the C-terminus of DdPLC from residue 669 to 736. In *Dictyostelium* lysates PLC activity is associated with the membrane, but we have evidence that a Ca^{2+}-dependent translocation process is not involved in regulating DdPLC (A. L. Drayer and P. J. M. van Haastert, unpublished work). In addition, some PLC isoforms, including *Dictyostelium* PLC, contain a second Ca^{2+}-binding site in an EF-hand motif. The purpose of this study was to investigate the role of the EF-hand domain in Ca^{2+}-dependent PLC activity.

The cloning of PLC from the cellular slime mould *D. discoideum* and the isolation of mutants with a disrupted *plc* gene containing no PLC activity provided an ideal system for expression of mutated PLC proteins in their native environment. For the experiments described here, we constructed an independent *Dictyostelium plc*⁻ mutant by disruption of the en-

dogenous DdPLC gene. This new *plc*$^-$ strain DH1.19 confirmed the results obtained for the *plc*$^-$ strain HD10 [14] that DdPLC accounts for all PLC activity in *Dictyostelium* and that *plc*$^-$ cells are not affected in growth or development. Expression of DdPLC in the *plc*$^-$ mutant from a highly active promoter resulted in cells expressing high levels of PLC protein and PLC activity. These cells to which PLC activity was restored showed a normal phenotype as was previously observed for wild-type cells overexpressing DdPLC [12].

In EF-hand Ca^{2+}-binding sites, the Ca^{2+} ion is usually co-ordinated by seven ligands. We expected that removal of the co-ordinating oxygen atoms by site-directed mutagenesis would reduce the affinity of the enzyme reaction. In contrast, we observed that the Ca^{2+} concentration giving half-maximal activity was unaltered, whereas the maximal activity decreased markedly. We also observed that the Ca^{2+}-activation curves were steeper for mutants with multiple mutations, as represented by the Hill coefficient. We tried to demonstrate direct binding of Ca^{2+} to unmutated DdPLC using purified recombinant DdPLC in a $^{45}CaCl_2$ overlay assay [42]. After SDS/PAGE and blotting of proteins to nitrocellulose membranes we were unable to detect Ca^{2+} binding to DdPLC, although in our hands Ca^{2+}-binding to α-actinin [43] was detected under these conditions (M. E. Meima and A. L. Drayer, unpublished work). It is therefore not resolved whether the reduced maximal PLC activity in the EF-hand-mutated proteins is caused by loss of Ca^{2+} binding to the EF-hand.

The first residue in the Ca^{2+}-binding loop of an EF-hand is an invariant aspartate, which is considered to play an essential role in binding of the Ca^{2+} ion [44]. We expected that if the EF-hand of DdPLC is a true Ca^{2+}-binding regulatory site, mutation of this first residue in the Ca^{2+}-binding loop would have a profound effect on enzyme activity. Our Ca^{2+}-activation data demonstrate that replacement of this aspartate with an amino acid without an oxygen-containing side chain had little effect on the Ca^{2+} affinity and maximal activity. Although the Ca^{2+}-binding-loop sequence was completely altered by introducing additional mutations, the affinity for Ca^{2+} did not decrease. In contrast, PLC activity at saturating Ca^{2+} concentrations was affected by the mutations. The different mutated proteins showed a progressive reduction in maximal activity with increasing number of mutations, with 40% activity after two mutations, 15% activity after three mutations and 8% activity after five mutations.

Although the putative EF-hand Ca^{2+}-binding site is required for PLC activity, the results clearly show that this site in *Dictyostelium* PLC does not regulate the Ca^{2+}-dependence of the enzyme reaction. In *Dictyostelium* PLC, the EF-hand is situated between the conserved A and B domains in a region containing 12 acidic amino acid residues. This region between the putative catalytic domains also contains a high percentage of acidic residues in PLC-β and PLC-δ isoforms. Limited proteolysis of PLC-δ1 suggests that this hydrophilic region is exposed, forming a loop to connect the A and B domains in the catalytic core [17,22]. It is possible that the Ca^{2+}-dependence of PLC resides in either the interaction between substrate and enzyme, for instance through the Ca^{2+}-dependent phospholipid-binding domain, or the interaction between Ca^{2+} and the substrate, through formation of a Ca^{2+}–PtdIns(4,5)P_2 complex. The putative EF-hand domain could fulfil a more structural role to bring together the A and B domains of *Dictyostelium* PLC to form the correct active structure.

In summary, expression of *Dictyostelium* PLC mutants in their natural cellular context reveals that the putative EF-hand Ca^{2+}-binding domain is essential for enzyme activity, but does not mediate the Ca^{2+}-dependence of the PLC enzyme reaction.

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