Acta Biochimica Polonica

Vol. 44 No. 2/1997

221–230

QUARTERLY

Expression, purification and kinetic properties of human recombinant phospholipase C δ3*

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Received: 27 February, 1997; accepted: 10 May, 1997

Key words: phospholipase C δ3, expression, purification, calcium, polyamines, basic proteins

To obtain sufficient quantities of pure phospholipase C δ3 (PLC δ3) necessary for structural and kinetic studies, cDNA of human fibroblast PLC δ3 was cloned in the pPROEX-1 vector, expressed in *E. coli* cells as a (6 × His) fusion protein and purified to homogeneity. From 1 L of *E. coli* culture 8 mg of pure PLC δ3 was obtained by a two step purification procedure, which includes phosphocellulose and Mono S cation exchange chromatography.

The presence of His tag did not affect the catalytic and regulatory properties of PLC δ3. The K_{app} for PIP_2 was 142 ± 11 and 156 ± 12 μM for His.PLC δ3 and PLC δ3, respectively. Recombinant PLC δ3 showed an absolute requirement for Ca^{2+}. Increasing the free Ca^{2+} concentration from 0.2 to 0.5 μM resulted in a sharp increase in enzyme activity. In comparison with human recombinant PLC δ1 the δ3 isoenzyme was more sensitive to low Ca^{2+} concentration. The Ca^{2+} concentration yielding maximal activation of PLC δ1 and PLC δ3 was 10 and 1 μM, respectively. The activity of PLC δ3 was stimulated by polyamines and by basic proteins such as protamine, histone and mellitin. PLC δ3 was activated most effectively by spermine and histone but the extent of this activation was lower than for PLC δ1.

The data presented indicate that the expression of PLC δ3 in *E. coli* cells permits to obtain active enzyme. The catalytic and regulatory properties of PLC δ3 are similar to those of PLC δ1.

Diacylglycerol and 1,4,5-triphosphate (IP_3) are the products of phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis catalyzed by phospholipase C (PLC). The generated diacylglycerol and IP_3 cause a rise in activity of protein kinase C and in cytosolic Ca^{2+} concentration, respectively [1, 2]. Thus, changes in Ca^{2+} and diacylglycerol affect virtually every aspect of cellular regulation either directly or indirectly. Therefore, control of PLC activity is one of the major entry points into cellular regulation.

*This work was supported by the State Committee for Scientific Research (KBN) grant No. 6 P207 090 06 to T.P.

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Abbreviations: PLC, phospholipase C; PIP_2, phosphatidylinositol-4,5-bisphosphate; PIP, phosphatidylinositol-4-phosphate; IP_3, inositol-1,4,5-triphosphate; IPTG, isopropyl β-D-thiogalactopyranoside, PEG, polyethylene glycol.

Three major types of phosphoinositide-specific phospholipase C named β, γ and δ have been characterized [3, 4]. The different isoforms of PLC are regulated differently. PLC γ appears to be regulated by tyrosine phosphorylation in response to epidermal growth factor, fibroblast growth factor, nerve growth factor and platelet-derived growth factor receptor occupancy [5]. The phosphorylation of PLC γ does not affect the kinetic properties of the enzyme, but causes a redistribution of the enzyme from cytosol to cell membrane [6, 7]. Tyrosine phosphorylation of PLC γ promotes its association with actin components of the cytoskeleton [8, 9]. PLC β isoenzymes are activated by the α and βγ subunits of the heterotrimeric G proteins [10–12]. The receptors that are known to activate PLC β *via* α subunit of Gq are those for bradykinin, angiotensin II, thromboxane A2, vasopressin and acetylcholine [10]. The receptors for interleukin-8 and m2 and m4 subtypes of muscarinic acetylcholine receptor activate PLC β by βγ subunits of G proteins [10, 13–15]. The regulatory mechanisms of PLC δ isoenzymes (δ1, δ2, δ3, δ4) are not known at the present time. Recently Homma & Emori [16] reported that PLC δ1 binds to a novel GAP (GTPase activating protein) specific for Rho. This suggests that the Rho pathway may be involved in the regulation of PLC δ1. The regulation of PLC δ1 *in vitro* critically depends on phospholipids, polyamines and calcium [17–21]. As part of our continuing effort in investigating the regulatory mechanism for δ isoforms of PLC we examined the catalytic and regulatory properties of human fibroblast PLC δ3 which, we have expressed in *Escherichia coli* cells and purified to homogeneity.

MATERIALS AND METHODS

Crude phosphoinositide mixture, spermine, leupeptin, isopropyl β-D-thiogalactopyranoside, and carbenicillin, were from Sigma-Aldrich Sp. z o.o. (Poznań, Poland). Pefabloc SC was from Boehringer Mannheim GmbH Biochemica (Mannheim, Germany). [2-^{3}H]Inositol-PI-4,5-P_2 (4.8 Ci/mmol) was purchased from Du Pont GmbH (Dreieich, Germany). pPROEX-1 vector, Ni^{2+}-NTA resin and rTEV protease were from Gibco BRL (Gaithersburg, MD, U.S.A.). Phosphocelulose P-11 was from Whatman (Maidstone, Kent, U.K.). BioLogic System (FPLC) was from, Bio-Rad Laboratories Ges.m.b.H., Wien, Austria. Mono S column was from Pharmacia Biotech Ges.m.b.H., Wien, Austria.

Unlabelled PIP_2 was purified from a crude phosphoinositide mixture by thin-layer chromatography according to Jolles *et al.* [22]. This method was also used for analytical separation of PIP_2 and PIP. Phospholipid concentrations were determined after perchloric acid digestion [23] by measuring orthophosphate [24]. Protein was determined by Bradford's method [25] using bovine serum albumin as a standard, or by measuring the absorbance at 280 nm.

The cDNA for PLC δ3 from human fibroblasts was originally cloned into pBluescript II SK (Stratagene, La Jolla, CA, U.S.A.) by Bristol *et al.* [26]. An *Nde*I site was introduced at the beginning of the coding sequence of PLC δ3 (Ghosh, S., Pawełczyk, T. & Lowenstein, J.M., unpublished). The cDNA of PLC δ3 was then cloned into the unique *Nde*I and *Bam*HI sites in pPROEX-1. pPROEX-PLCδ3 was used for the expression of PLC δ3 as a fusion protein with the 6 × His fragment at the N-terminus. For the expression of His.PLC δ3 the BL21(DE3) *E. coli* strain (Novagen, Madison, WI, U.S.A.) was used.

Expression of PLC δ3. BL21(DE3) cells transformed with pPROEX-PLCδ3 were grown in 1 L of LB medium containing carbenicillin 50 μg/mL at 37°C with vigorous shaking until A_{600} reached 0.95. Cells were then cooled to 24°C and expression was induced by adding IPTG to a final concentration of 0.5 mM. Cells were cultured for an appropriate time and harvested at 5000 × ***g*** in a Beckman centrifuge for 10 min.

Purification of PLC δ3. *E. coli* cells from 1 L culture were suspended in 20 mL of 50 mM potassium phosphate buffer, pH 6.0, 300 mM KCl, 10 mM 2-mercaptoethanol, 0.2 mM Pefabloc SC, 20 μM leupeptin, 20% glycerol (hereafter referred to as buffer A) and sonicated on dry ice for five 1 min bursts with 1

min intervals for cooling (care being taken not to freeze the sample). All subsequent steps were carried out at 0–4°C. The crude extract was centrifuged at 50000 × *g* for 30 min. The resulting supernatant was applied to a column containing 3 mL of Ni^{2+}-NTA resin pre-equilibrated with buffer A. The column was washed with 50 mL of buffer A and eluted with the same buffer containing 100 mM imidazole. Alternatively, the supernatant obtained by centrifugation at 50000 ×*g* was mixed with phosphocellulose (1 g wet wt./1 mL) which had been equilibrated with buffer A. The slurry obtained was sedimented by centrifugation at 5000 × *g* and supernatant was discarded. The pellet was washed by mixing with buffer A and centrifuged as above. Supernatant was discarded, the pellet was suspended in buffer A containing 0.6 M KCl and the mixture was centrifuged. The supernatant or the pooled fractions from Ni^{2+}-NTA column containing His.PLC δ3 were desalted on Sephadex G-25 equilibrated with 50 mM potassium phosphate buffer, pH 6.0, 1 mM dithiothreitol, 0.2 mM Pefabloc SC, 20 µM leupeptin, 20% glycerol. Desalted His.PLC δ3 was purified by FPLC on a Mono S column as described previously [19]. Fractions containing His.PLC δ3 were pooled, transferred to a dialysis bag, placed on PEG 40000 and concentrated to a protein concentration of 5.0 mg/mL. The His tag was removed from PLC δ1 by incubation of the enzyme with rTEV protease (60 µg protein/5 U rTEV) at 4°C for 24 h. Finally, the pH of the reaction mixture was lowered to 6.0 by adding 0.5 M KH_2PO_4 and the PLC δ3 was separated from rTEV protease by chromatography on a Mono S column.

Assay for PLC δ3. PLC δ3 activity was assayed as described in detail previously for PLC δ1 [17]. The reaction mixture contained 17 nmol/mL [2-^{3}H]inositol-labelled phosphatidylinositol-4,5-bisphosphate (1200–1400 d.p.m./nmol), 2.4 mM sodium deoxycholate, 180 mM NaCl, 100 µM $CaCl_2$, 100 µM EGTA, 50 mM Hepes-NaOH buffer, pH 7.2.

The reaction was started by adding PLC δ3 and was run in a final volume of 0.1 mL at 37°C for 1 min. The concentration of free Ca^{2+} in the reaction mixture was 2.2 µM as determined by the arsenazo method [27]. The reaction was stopped by adding 0.1 mL of 1.2 N HCl, the mixture was vortexed, 0.5 mL chloroform-methanol (2:1) was added, and the mixture was vortexed again. The aqueous layer was separated, and an aliquot was taken for counting. Assay conditions were chosen so that the reaction rate was proportional to time and enzyme concentration. In the assay used neither the substrate nor the inhibitor was in true solution. For this reason we prefer to quote their concentrations as nmol/mL. If substrate and inhibitor were in true solution, nmol/mL would become µM. One unit (U) of enzyme activity was the enzyme activity that hydrolyzed 1 µmol of PIP_2 in 1 min at 37°C.

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecylsulfate (SDS) was performed according to Laemmli [28]. Protein bands were developed by Coomassie Brilliant Blue staining.

RESULTS

To obtain large quantities of active PLC δ3 we have used the polyhistidine (6 × His) fusion gene vector system. The cells transformed with pPROEX-PLC δ3 were induced in the late log phase (A_{600} = 0.95) because we observed the highest production of the enzyme when IPTG was added at an A_{600} between 0.8 and 1. The PLC δ3 protein production increased with lowering of the induction temperature from 37°C to 22°C. Further reduction in the temperature to 16°C provided a higher yield of the soluble protein than that obtained at 22°C; however, induction times were longer. Maximal activity of PLC δ3 in cell lysates from 1 L culture grown at 37, 22 and 16°C was obtained at 8, 18 and 72 h and was 65, 850 and 3200 units, respectively. As the most convenient conditions for the expression of PLC δ3 we have chosen to use the induction time of 18 h and the temperature 22°C.

The process of His.PLC δ3 purification started with 5 g of *E. coli* cells obtained from 1 L of the cell culture. A soluble bacterial lysate was first subjected to batch separation on phosphocellulose. Subsequent chromatog-

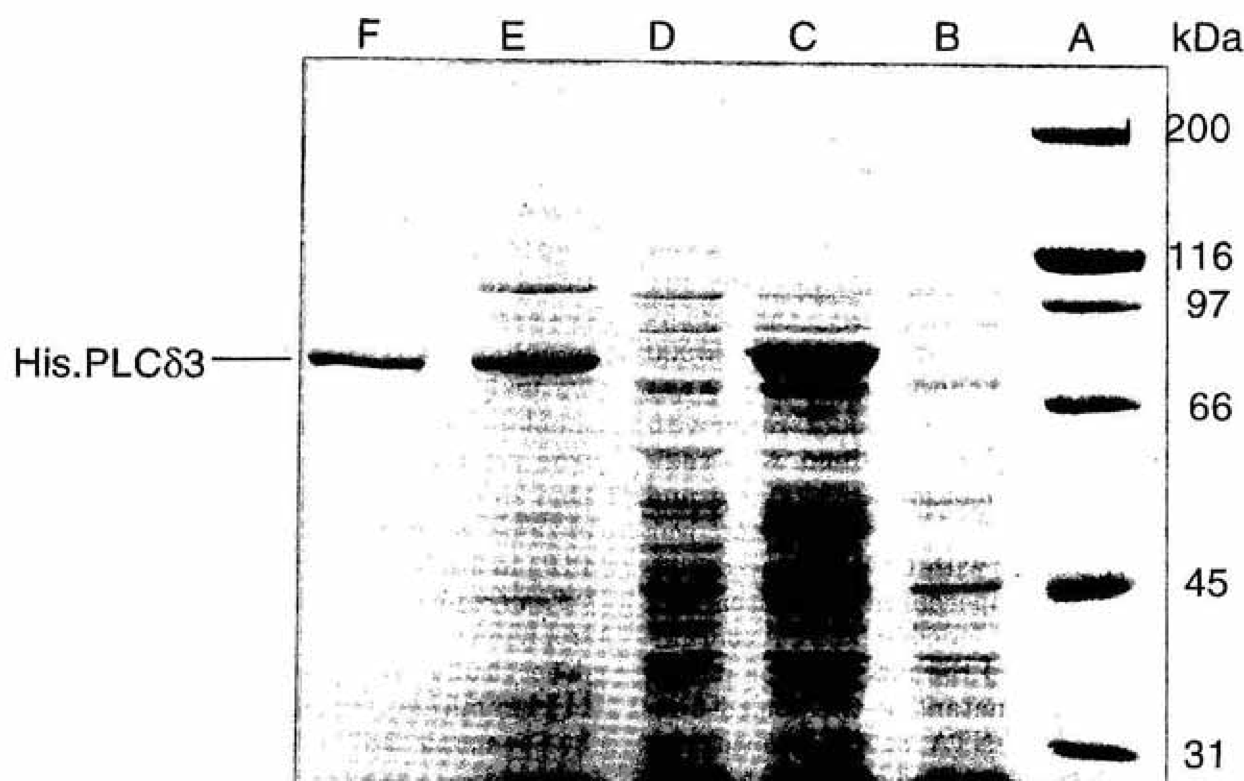

Figure 1. Eight percent SDS-polyacrylamide gel electrophoresis of human recombinant phospholipase C δ3.

Lane A, molecular mass standards; lane B, 50 μg of *E. coli* cells lysate (not induced); lane C, *E. coli* cells lysate induced for 18 h at 22°C; lane D, 50 μg of *E. coli* extract (Table 1); lane E, 7 μg of His.PLC δ3 purified on phosphocellulose (Table 1); lane F, 2 μg of His.PLC δ3 purified on Mono S cation exchanger column (Table 1). The results are representative of those obtained in nine separate experiments.

raphy on cation exchange (Mono S) column gave electrophoretically pure His.PLC δ3 (Fig. 1). Removal of the His tag was performed by incubation of His.PLC δ3 with rTEV protease at 4°C for 24 h. The specific activity of purified PLC δ3 was 53 U/mg. This represented a 26-fold purification with 50% yield (Table 1). SDS gel electrophoresis of purified PLC δ3 showed a band of 83 kDa (Fig. 1). This is in good agreement with the calculated mass of 83581 Da. The advantage of using His tagged protein is the ease of purification on metal-affinity column. However, we discovered that the purification of His.PLC δ3 on phosphocellulose and Ni^{2+}-NTA resin gave almost identical results (Fig.

Table 1. Purification of recombinant human phospholipase C δ3.

The purification started with 5 g of *E. coli* cells obtained from 1 litre of 18 h cell culture grown at 22°C. Phospholipase C δ3 was assayed as described in Materials and Methods. One unit (U) is the amount of enzyme that hydrolyses 1 μmol of PIP_2 in 1 min under the conditions employed. The data presented are representative of those obtained in seven separate purifications.

Step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)
E. coli extract	421	850	2.02	100
Phosphocellulose	45	510	11.33	60
Mono S	8	425	53.13	50

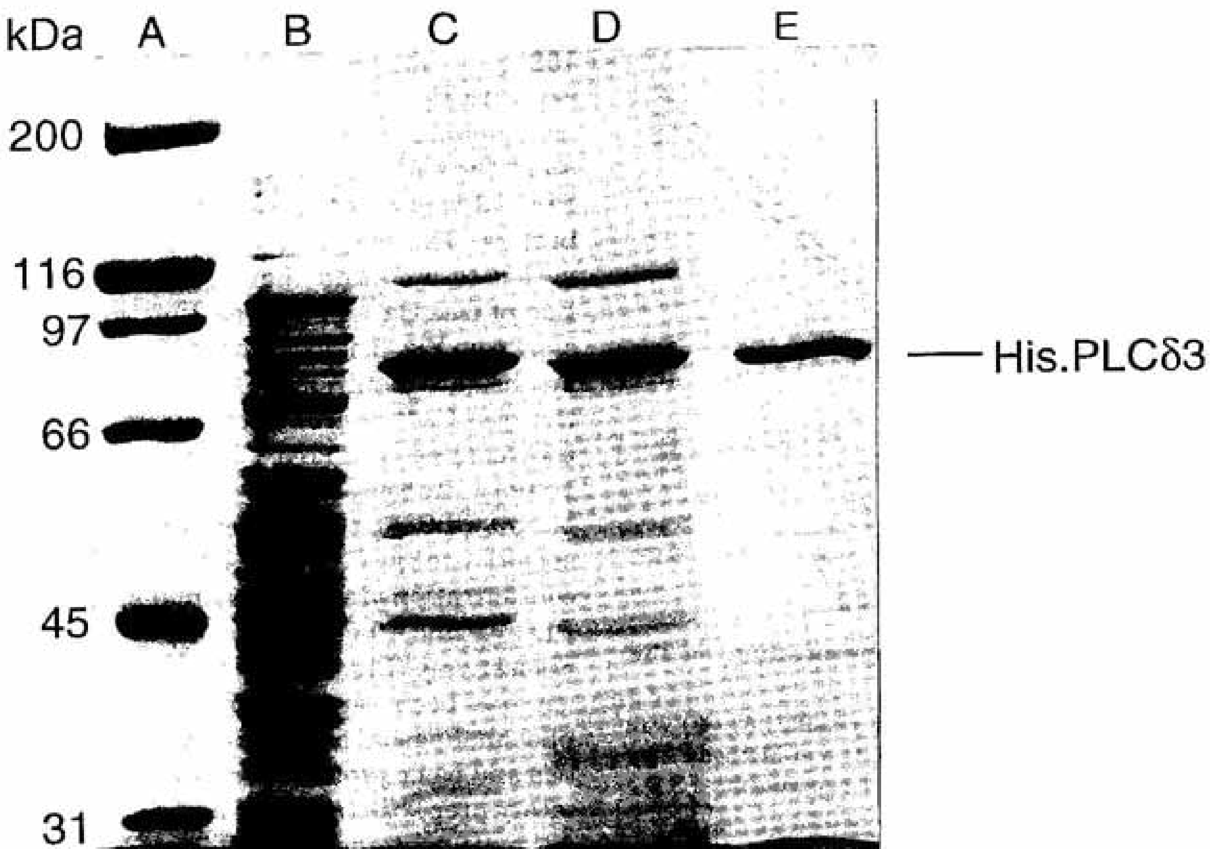

Figure 2. Comparison of the efficiency of His.PLC δ3 purification on Ni-NTA resin and phosphocellulose.

The samples were electrophoresed on 8% SDS-polyacrylamide gel. Lane A, molecular mass standards; lane B, 50 μg of *E. coli* extract (Table 1); lane C, 7 μg of His.PLC δ3 purified on Ni-NTA resin; lane D, 7 μg of His.PLC δ3 purified on phosphocellulose; lane E, 2 μg of His.PLC δ3 purified on Mono S cation exchanger column.

2). Therefore, for the routine purification we have chosen the phosphocellulose batch separation as more cost effective. The advantage of the use of 6 × His fusion gene vector system was that the expressed His.PLC δ3 was less sensitive to degradation by proteases than PLC δ3. Therefore, the yield of the His.PLC δ3 purification was much higher than that of PLC δ3 (data not shown). To examine the stability of His.PLC δ3 and PLC δ3 we incubated the enzyme with rat liver cytosol (Fig. 3).

Comparison of His.PLC δ3 and PLC δ3 activity revealed no change in the activity

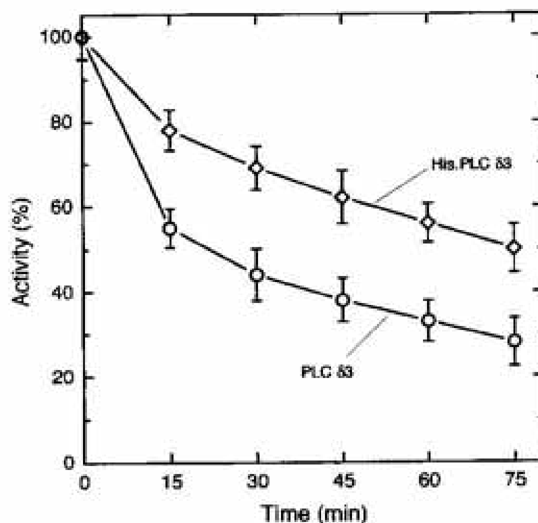

Figure 3. The activity of PLC δ3 incubated with rat liver cytosol.

The purified PLC δ3 (1 μg) and His.PLC δ3 (1 μg) were incubated at 24°C for the time indicated in 0.1 ml of 50 mM Hepes/NaOH buffer, pH 7.2, containing 100 mM NaCl and 2 mg of rat liver cytosol protein. The activity of PLC δ3 and His.PLC δ3 at 0 time, 7.43 and 6.78 nmol/min per mL, respectively was taken as 100%. The results are mean values from three experiments ± S.D.

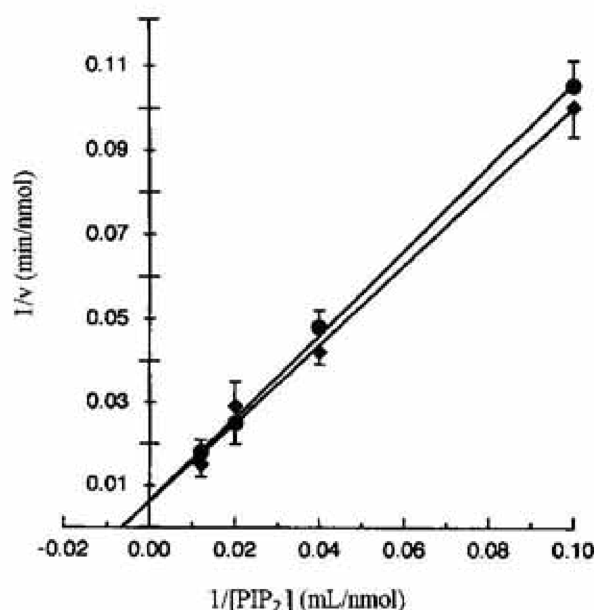

Figure 4. Effect of PIP_2 concentration on the enzyme activity.

The activity of PLC δ3 (●) and His.PLC δ3 (◆) was assayed as described in Materials and Methods. The results are mean values from five experiments ± S.D.

and regulatory properties of the enzyme upon removal of His tag (Fig. 4). The K_{app} for

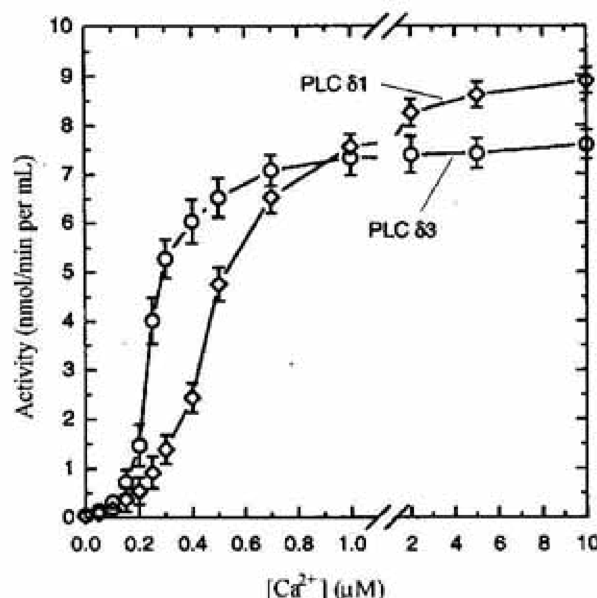

Figure 5. The effect of Ca^{2+} concentration on the activity of recombinant PLC δ3 and PLC δ1.

The activity of purified PLC δ1 and PLC δ3 was assayed as described in Materials and Methods. Free $[Ca^{2+}]$ was varied by changing the proportion of $CaCl_2$ to EGTA in the calcium buffer. The concentrations of free $[Ca^{2+}]$ was calculated using the SPECS program [36]. The results are mean values from five (recombinant PLC δ3) and seven (recombinant PLC δ1) experiments ± S.D.

PIP_2 in the detergent assay was 142 ± 11 and 156 ± 12 μM for His.PLC δ3 and PLC δ3, respectively. All PLC's required Ca^{2+} for phosphoinositides hydrolysis. The requirement for Ca^{2+} in the reaction catalyzed by PLC δ3 was investigated using EGTA/Ca^{2+} buffers. The ratios of Ca^{2+} to EGTA were varied to maintain the required concentration of free Ca^{2+} in the reaction mixture. As shown on Fig. 5, recombinant PLC δ3 showed an absolute requirement for Ca^{2+}. Increasing the free Ca^{2+} concentration from 0.2 to 0.5 μM resulted in a sharp increase in enzyme activity. In comparison with human recombinant PLC δ1 the δ3 isoenzyme was more sensitive to low Ca^{2+} concentration. The Ca^{2+} concentration yielding maximal activation of PLC δ1 and PLC δ3 was 10 μM and 1 μM, respectively (Fig. 5).

It has been reported that the activity of phospholipase C δ1 is stimulated by polyamines and basic proteins. To compare the regulatory properties of the δ1 and δ3 isoenzymes we have examined the effect of polyamines and such basic proteins as protamine, histone and mellitin on the activity of recombinant PLC δ3. PLC δ3 was activated most effectively by spermine and histone but the extent of this activation was lower than for PLC δ1 (Table 2).

DISCUSSION

This is to our knowledge the first report describing the catalytic and regulatory properties of phospholipase C δ3. The content of PLC δ3 in mammalian tissues is very low. This could be the reason that our initial attempts at PLC δ3 purification from various tissues were unsuccessful. The system for expression of PLC δ3 presented in this contribution allows to obtain high quantities of the active enzyme. From 1 L of 18 h *E. coli* culture 8 mg of pure enzyme was obtained by a two step purification procedure (Table 1).

Previously we have reported that the activity and regulatory properties of human fibroblast PLC δ1 expressed in *E. coli* were almost identical to those of the enzyme purified from rat liver despite the different expression system and animal and tissue

Table 2. The effect of basic proteins and polyamines on the activity of human recombinant PLC δ1 and PLC δ3.

The activity of purified PLC δ1 [21] and PLC δ3 was assayed as described in Materials and Methods. One hundred percent of PLC δ1 and PLC δ3 activity was 1.12 and 1.82 nmol/min per mL, respectively. The results are mean values from three experiments ± S.D.

Effector	Concentration	Activation (% of control)	
	(μg/mL)	PLC δ1	PLC δ3
Histone	5	307 ± 10	218 ± 8
Melittin	5	280 ± 9	119 ± 10
Lysozyme	50	202 ± 8	53 ± 7
	(mM)		
Spermine	0.15	650 ± 11	141 ± 9
Spermidine	1.0	410 ± 9	96 ± 11
Putrescine	10	330 ± 7	40 ± 6

source [20, 21]. Since the post-translational modification system in *E. coli* is different from the eukaryotic system, it is possible that the recombinant enzyme is structurally different from the purified one. The results obtained by us suggest that lack (if any) of post-translational modifications that are specific for mammalian cells does not play an important role for the catalytic and regulatory properties of recombinant PLC δ1 [21]. We assumed that, similarly to PLC δ1 the δ3 isoenzyme is expressed in *E. coli* as a fully functional enzyme and should be suitable for *in vitro* regulatory analyses.

The activity and regulatory properties of human fibroblast PLC δ3 expressed in *E. coli* appear to be similar to those of the human recombinant PLC δ1 [21]. In general PLC δ3 is less active than PLC δ1. The K_{app} for PIP_2 is about five times higher for PLC δ3 than for PLC δ1. The relationship between calcium concentration and the enzymatic activity is very similar for PLC δ3 and PLC δ1 [21, 29]. On the other hand, PLC δ3 is less sensitive to stimulation by polyamines and basic proteins (Table 2).

The various γ and β isoforms of PLC appear to be activated by different mechanisms but the regulatory mechanism for δ isoforms is not known at present. Four mammalian PLC δ (PLC δ1, PLC δ2, PLC δ3, PLC δ4) have to date been characterized on the basis of cDNA and amino-acid sequences [5, 30, 31]. The data on catalytic and regulatory properties of PLC δ isoforms reported to date indicate that all δ isoforms preferentially hydrolyse PIP_2 and are dependent on Ca^{2+} [21, 30, 31]. The reported K_m values for PIP_2 are similar for all of them [21, 30, 31]. Polyamines and basic proteins stimulate the δ1 as well as the δ3 isoform ([18] and Table 2). This rises the question whether there exist specific mechanisms that regulate particular δ isoforms of PLC. Recently it has been reported that the content of PLC δ4 increases dramatically at S-phase of the cell cycle and that this isoform is located in nucleus [32]. During the cell cycle, the level of PLC δ1 which is mostly located in the cytosol, was not changed. On the other hand, an increase in the activity of PLC δ1 was reported in aortas of spontaneously hypertensive rats [33]. Abnormal accumulation of PLC δ in Alzheimer brains [34] and other neurodegenerative diseases have been reported [35]. The data available to date suggest that the δ isoforms of PLC respond to factors that induce changes in a long term. In future may be of interest to identify the relationship between the changes in the activities of different δ isoforms of PLC and the changes in the polyamines and phospholipids cell content occurring during cell life.

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