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Rabbit fast skeletal muscle phospholipase C

Molecular weight determination by renaturation after polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate

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Phosphoinositide specific phospholipase C from rabbit fast skeletal muscle has been enriched ca. 1,000-fold with a specific activity of 40 μmol × min^{-1} × mg^{-1}. Following SDS-PAGE, renaturation of the enzyme protein in the presence of deoxycholate allowed the determination of an apparent molecular weight of 110 kDa. Gel-filtration of the native enzyme resulted in a very similar apparent molecular weight of 115 kDa, however, associated proteins of higher molecular weight were also found. Free Ca^{2+} concentrations needed for half-maximal activation of PtdIns(4,5)P_2, PtdIns4P and PtdIns hydrolysis are 6.3 μM, 85 μM and 1.8 mM, and the K_m values for these substrates 102, 340 and 937 μM, respectively.

Phospholipase C; Fast skeletal muscle; Molecular weight determination; Renaturation

1. INTRODUCTION

In the resting state, the myoplasmic Ins(1,4,5)P_3 concentration in various vertebrate muscle ranges from 1.2 to 2.5 μM; upon tetanic stimulation its concentration increases up to 4.2 μM within ca. 10 s. Therefore, it has been postulated that phosphoinositide-specific phospholipase C (PLC), which generates two second messengers, Ins(1,4,5)P_3 and diacylglycerol, from phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) is activated by membrane depolarization [1]. It is, however, unknown how these two events, depolarization and PLC activation, could be coupled. In fast skeletal muscle, the activity of PLC is as high as in other tissues [2,3]. The enzyme has been shown to be associated specifically with T-tubules, behaving like a peripheral membrane protein and completely inactive below ca. 10^{-7} M free calcium [2].

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Abbreviations: Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; PtdIns, 1-(3-*sn*-phosphatidyl)-D-*myo*-inositol; PtdIns(4)P, 1-(3-*sn*-phosphatidyl)-D-*myo*-inositol-4-phosphate; PtdIns(4,5)P_2, 1-(3-*sn*-phosphatidyl)-D-*myo*-inositol-4,5-bisphosphate; PtdSer, (3-*sn*-phosphatidyl)-L-serine; PtdEtn, (3-*sn*-phosphatidyl)ethanolamine; DOC, deoxycholate; SDS, sodium dodecylsulfate.

Two major signalling pathways leading to activation of PLC have been characterized. The β isoform seems to couple to several cell surface receptors via specific G proteins called Gq. This kind of PLC activation apparently can occur in slow oxidative muscle in which prazosine, an α_1 receptor agonist, stimulates Ins(1,4,5)P_3 formation and degradation 5- to 8-fold [4]. However, in fast skeletal muscle α_1 receptors are essentially absent [2]. Alternatively, the SH_2 domain containing isoform γ is activated by growth factor receptors involving phosphorylation of a tyrosine residue. The time-course of tyrosine phosphorylation can be correlated with the Ins(1,4,5)P_3 production rate. This suggests that phosphorylation of PLC is probably responsible for stimulation of phosphoinositide breakdown. Again, this type of PLC activation has not been demonstrated to occur in skeletal muscle.

Other signalling pathways may feed into these two basic PLC-activating mechanisms: elevated 3′5′-cyclic AMP levels might modulate phospholipase C activity, and protein kinase C has been reported to phosphorylate, in vitro, the PLC isoforms, β_1, γ and δ without changing their enzymatic activity.

In order to clarify the signalling pathway for PLC activation in skeletal muscle, the enzyme of this tissue must be characterized, which has not been carried out yet. In the present study, we describe an enrichment of PLC from rabbit fast skeletal muscle employing the high resolving power of polyacrylamide gel electrophoresis in the presence of SDS coupled with a new renaturation assay.

2. MATERIALS AND METHODS

2.1. *Enrichment of PLC from rabbit fast skeletal muscle*

100 g of skeletal muscle was homogenized in 300 ml buffer A (50 mM Tris/HCl, 1 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, 1 mM benzamidine and 1 μM leupeptin, pH 7.5) and the homogenate centrifuged at 8,000 × *g* for 30 min. The resulting crude extract was saturated up to 25% with solid ammonium sulfate; after removal of the precipitate the enzyme was collected by further saturation up to 60% ammonium sulfate and centrifugation at 30,000 × *g* for 20 min. The pellet was dissolved in and dialyzed overnight against 20 mM Tris-HCl, 1 mM EDTA, 0.5 mM EGTA, pH 7.5 (buffer B) containing 1 mM PMSF, 1 mM benzamidine and 1 μM leupeptin. The dialyzate was applied to DEAE-Sepharose CL-6B (5 × 30 cm) equilibrated with buffer B (flow rate 120 ml/h). PLC was eluted with buffer B containing additionally 1 M KCl (buffer C) using the following gradient programme: 0–3,400 ml, 0–50% C, 3,400–3,500 ml, 50–100% C, 3,500–5,000 ml 100% C. A first activity peak appeared at ca. 150 mM, and a second one at ca. 250 mM KCl. The enzymatic activity of the first pool was labile; storage at +4°C resulted in 80% loss within two days. Fractions containing the second activity peak were pooled and adjusted to 1 mM PMSF, 1 mM benzamidine, 1 μM leupeptin, 4 μg/ml calpain inhibitor I and 4 μg calpain inhibitor II. The solute was applied to hydroxyapatite (2.6 × 20 cm) pre-equilibrated in 20 mM KH_2PO_4/K_2HPO_4, 1 mM EDTA, 0.5 mM EGTA, pH 6.8 (buffer D; flow rate 45 ml/h). The enzyme was eluted with a linear gradient of 400 mM KH_2PO_4/K_2HPO_4, 1 mM EDTA, 0.5 mM EGTA, pH 6.8. Activity-containing fractions were pooled and brought to 60% ammonium sulfate. The precipitate collected by centrifugation at 30,000 × *g* for 20 min was dissolved in a small volume of buffer D and dialyzed against the same buffer overnight. Subsequently, chromatography on phosphocellulose (2.6 × 20 cm) was carried out under the same buffer and elution conditions (flow rate 30 ml/h) as described for hydroxyapatite.

The PLC activity-containing pool was applied to Heparin–Sepharose (1.6 × 25 cm) pre-equilibrated in buffer B (flow rate 60 ml/h). The enzyme was eluted with buffer B containing 1.5 M KCl (buffer E) employing the following gradient: 0–400 ml 0% E; 400–750 ml 0–100% E; 750–800 ml, 100% E. Activity-containing fractions were pooled and applied directly onto phenyl–Sepharose (1 × 13 cm) pre-equilibrated with buffer B containing 500 mM KCl (buffer F; flow rate 30 ml/h). PLC was eluted with buffer B containing 2% deoxycholate (DOC) (buffer G) employing the following gradient: 0–20 ml, 0% B, 20–40 ml, 0–100% B; 40–50 ml, 100% B; 50–110 ml, 100% B–100% G 110–120 ml 100% G.

The activity-containing fractions were pooled, dialyzed against buffer B and applied to Mono-Q (0.5 × 5 cm; HR 5/5) pre-equilibrated in buffer B (flow rate 30 ml/h). The enzyme was eluted with buffer F employing the following gradient: 0–5 ml, 0-20% F; 5–9 ml, 20% F; 9–18 ml, 20–100% F; 18–20 ml, 100% F. Activity-containing fractions were pooled and dialyzed against buffer B. The enzyme was stored at +4°C (ca. 10% activity loss over ca. 3 months).

2.2. *Determination of PLC activity*

PLC activity was assayed as described [2], using 676 μM PtdIns4P or 600 μM PtdIns4,5P_2 as substrates.

2.3. *Renaturation of PLC following polyacrylamide gel electrophoresis (PAGE) in the presence of SDS*

10% SDS-PAGE was carried out according to [5]. A sample of the phenyl–Sepharose pool was concentrated by AMICON ultrafiltration and ca. 40 μg protein was applied in SDS-sample buffer onto the polyacrylamide gel. Electrophoresis was carried out at +4°C overnight. The PLC-containing slot was cut in 2 mm segments which were incubated overnight by gently shaking at +4°C in 200 μl renaturation buffer consisting of 50 mM Tris-HCl, 1 mM EDTA, 0.5 mM EGTA, 0.14% DOC, 0.03% NaN_3 and 1 mg/ml bovine serum albumin (BSA), pH 7.2. PLC activity was assayed in supernatants obtained by centrifugation of the disrupted gel/renaturation buffer mixture. PtdIns(4,5)P_2 or PtdIns4P was used as the substrate; the incubation time was 17 h at 30°C. For detection of protein distribution a parallel slot was silver stained according to [6].

2.4. *Gel-filtration over Sephacryl S200*

Following concentration (AMICON PM10 filter) the material was applied to Sephacryl S200 superfine (2.6 × 95 cm) pre-equilibrated in 20 mM Tris-HCl, 1 mM EDTA, 0.5 mM EGTA, 400 mM NaCl, 0.03% NaN_3, pH 7.5. The gel-filtration column was calibrated with the following standards: ferritin (450 kDa), catalase (240 kDa), aldolase (158 kDa), BSA (68 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa), cytochrome *c* (12.5 kDa).

2.5. *Other methods*

Protein was determined by the method of [7] employing BSA as the standard.

^3H radioactivity was determined in Hydroluma (Baker Chemicals, Deventer, NL).

Calculations of free Ca^{2+} concentrations were carried out as described [8].

2.6. *Materials*

[^3H]PtdIns(4,5)P_2 and [^3H]PtdIns4P were purchased from BioTrend.

Table 1

Enrichment of PtdIns(4,5)P_2-PLC from rabbit skeletal muscle

Steps	Protein (mg)	Activity (μmol × min^{-1})	Specific activity (μmol × min^{-1} × mg^{-1})	Yield (%)	Enrichment (-fold)
Crude extract	50,502	1,785	0.035	100	1
Ammonium sulfate precipitate	23,650	1,766	0.075	99	2.1
DEAE–Sepharose pool 1	586	178	0.30	10	8.7
pool 2	1,160	580	0.50	32	14.3
Hydroxyapatite (pool 2)	367	411	1.12	23	32
Phosphocellulose	73	360	4.93	20	141
Heparin–Sepharose	25	253	10.1	14	289
Phenyl–Sepharose	4.0	79	19.8	4.5	564
Mono-Q	0.7	28	39.5	1.5	1,128

Details of PLC enrichment, determination of enzyme activity and protein are described in section 2.

PtdIns(4,5)P_2, PtdIns4P, PtdEtn and PtdSer were from Sigma. BioGel HTP hydroxyapatite was from Bio-Rad, phosphocellulose from Whatman, heparin-Sepharose, phenyl-Sepharose and Mono-Q were from Pharmacia LKB.

The molecular weight standards for SDS-PAGE were from Sigma. Protease inhibitors and proteins for calibration of the Sephacryl S200 column were from Boehringer-Mannheim. All other chemicals used were of p.A quality.

3. RESULTS

PLC from rabbit fast skeletal muscle has been enriched approximately 1,100-fold (overall yield 1.5%) by seven chromatographic steps on different ion exchange and hydrophobic materials (Table I). Repeatedly, a specific activity of ca. 40 μmol × min^{-1} × mg^{-1} was obtained.

Membrane bound PLC (ca. 10% of the total activity) is sedimented with microsomes by centrifugation at 100,000 × g. The enzyme can be solubilized with 600 mM KCl, and membranes are separated by re-centrifugation at 100,000 × g. This solubilized membrane bound PLC shows the same chromatographic behavior (not shown) as the enzyme demonstrated in Table I. Additionally, pH optima, kinetic parameters, Ca^{2+} concentration-dependent activation, and Mg^{2+}-induced inhibition and activation by DOC of both forms are identical within error of determinations.

At Ca^{2+} concentrations lower than 300 nM PLC activity is not detectable, either with PtdIns(4,5)P_2 or with PtdIns4P or PtdIns as substrates; half-maximal activation occurs at 6.3 μM, 85 μM and 1.8 mM Ca^{2+} for each of the above substrates, respectively. The K_m values for these three substrates are 102, 340 and 937 μM, respectively.

DOC influences the enzyme activity in a triphasic manner. Up to 1 mM the activity decreases to ca. 50%;

Table II

DOC protection of PLC activity against SDS inactivation

SDS (mM)	PtdIns(4,5)P_2-PLC activity (%)	DOC (mM)
0	100	0
0.05	95	0
0.10	32	0
0.50	0	0
0	193	3.5
0.05	165	3.5
0.10	100	3.5
0.50	21	3.5

PtdIns(4,5)P_2 hydrolysis was assayed by using PLC after phenyl-Sepharose chromatography as described in section 2.

further increase of [DOC] enhances the activity maximally 3.5-fold, corresponding to a ca. 2-fold activation of the activity in the absence of DOC: higher concentrations inhibit activity further (Fig. 1). SDS is a potent inhibitor of PLC; increasing concentrations up to 0.5 mM SDS completely abolish the activity (Table II). The optimally activating DOC concentration (3.5 mM, see Fig. 1) partially protects the enzyme against this SDS inhibition; in the presence of DOC a residual activity of 21% is observed at 0.5 mM SDS. This protecting activity of DOC can be employed to detect PLC activity following PAGE in the presence of SDS; an example is shown in Fig. 2. In the pherogram, a single PtdIns(4,5)P_2-PLC activity peak is seen exhibiting a M_r of 110 kDa. There is no difference when either PtdIns(4,5)P_2 or PtdIns4P is employed as the substrate. Silver staining of a parallel gel slot reveals a faint band of M_r 110 kDa, too (insert of Fig. 2).

An approximately identical M_r can be determined by

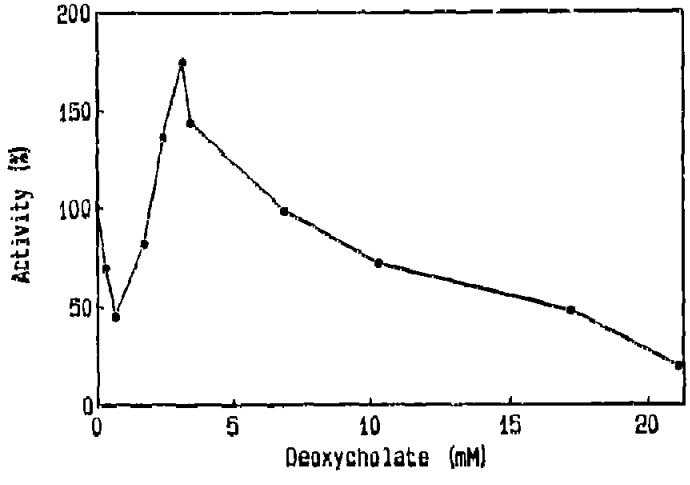

Fig. 1. Effect of deoxycholate on PLC activity. PtdIns(4,5)P_2 hydrolysis was assayed using PLC after phenyl-Sepharose chromatography as described in section 2. Deoxycholate in the concentration range tested does not influence the TCA precipitation of [^{3}H]PtdIns(4,5)P_2.

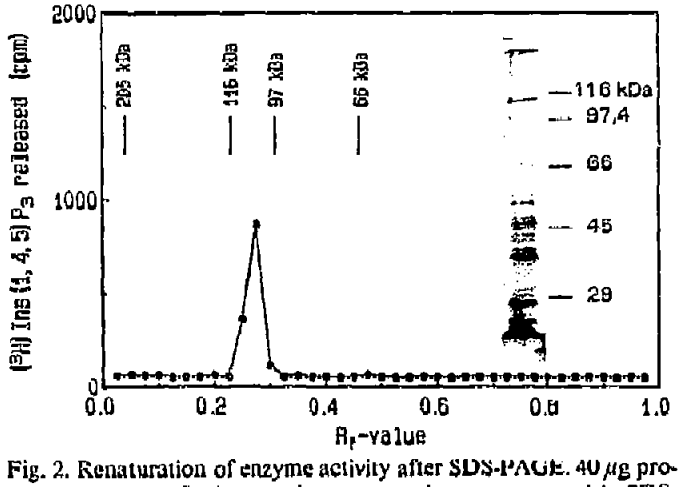

Fig. 2. Renaturation of enzyme activity after SDS-PAGE. 40 μg protein after phenyl-Sepharose chromatography was separated in SDS-PAGE. Renaturation of enzyme protein and the activity assays were carried out as described in section 2. (Insert) 5 μg protein after phenyl-Sepharose chromatography was separated in a parallel slot and the separated polypeptides were silver stained.

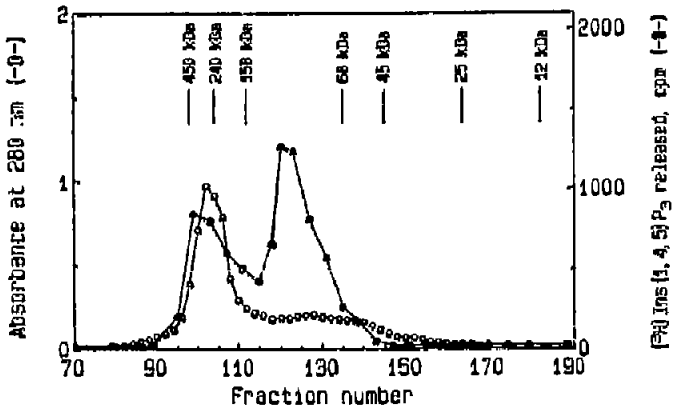

Fig. 3. Gel-filtration of DEAE pool 2 on Sephacryl S200. 32 mg DEAE pool 2 was applied to the gel-filtration column. Details of the chromatography, assays for enzyme activity and protein concentration are described in section 2.

gel filtration over Sephacryl S200. Two activity peaks are detectable: the first corresponding to a molecular weight of ca. 400 kDa, and the second corresponding to ca. 110 kDa (Fig. 3). The high molecular weight form was re-chromatographed on Sephacryl S200; the elution profile shows two activity maxima exhibiting again a M_r of ca. 400 and 110 kDa, respectively (not shown). Thus, the high molecular weight form might be an associated form of the enzyme from which an active monomeric 110 kDa form is released.

4. DISCUSSION

The rabbit hind leg and back muscles employed in these preparations consist of ca. 90% fast glycolytic and ca. 10% slow oxidative twitch fibres [9]. This material therefore is an excellent source for characterization of a fast skeletal muscle-type enzyme.

Upon chromatography of a 100,000 × *g* muscle supernatant on DEAE-Sepharose two PLC activity peaks are obtained. A minor activity peak eluting at low salt concentrations seems to be heterogeneous as it has been observed in subsequent gel-filtration experiments over Sephacryl S200, suggesting the existence of several isoforms (not shown). Due to its instability this fraction has not been further purified or characterized. Additionally, it might contain enzymes from other tissues than from fast skeletal muscle fibres. Therefore, the major and more stable PLC activity was studied further.

SDS gel electrophoresis of an enriched fraction and renaturation of the separated protein bands in DOC-containing buffer reveals a protein with an apparent M_r of 110 kDa which is in good agreement with the results obtained with the native enzyme by gel-filtration. Thus, the enzyme seems to be active as a monomeric species; associated forms, however, also seem to exist. In the SDS pherogram a protein band of M_r 110 kDa is also detectable; it represents ca. 3% of the total protein. The enriched enzyme has been purified ca. 1,100-fold; to obtain a homogeneous preparation, the PLC must be further purified ca. 30,000-fold, a range in which PLCs from other sources have to be purified [3]. The possibility of identifying the protein following SDS gel electrophoresis by the renaturation assay described here shortens the purification procedure considerably. Protein chemistry can then be carried out with the band excised from the gel.

A similar approach has been taken for renaturation of protein kinase activity following SDS gel electrophoresis and its subsequent detection by phosphorylation of substrates included in the polyacrylamide gel or of the kinase itself with [γ-^{32}P]ATP [10]. An alternative method is described by [11]; it is based on reducing the SDS concentration by dialysis in the presence of the neutral detergent, G3707. SDS can also be replaced by detergents like Triton X-100, octylglycoside or DOC [12].

In the case of rabbit skeletal muscle PLC, DOC was the most effective detergent at reactivating the SDS-inactivated enzyme. DOC enhances the enzymatic activity optimally at approximately the critical micelle concentration which is ca. 3 mM under the conditions of the renaturation assay chosen here. This renaturation procedure allows for the first time the identification of a protein band with PLC activity following SDS-PAGE employing different subcellular fractions or different samples obtained upon partial purification.

Only two PLCs with similar apparent molecular weights of ca. 110 kDa determined here for the skeletal muscle enzyme have been described: one from mast cells and one from bovine spermatozoes [3]. The molecular architecture of these 110 kDa forms is not known.

By Northern blot analyses indications for the presence of PLCδ (85 kDa) and PLCγ (145 kDa) in rat skeletal muscle could be found [13]. Rhee et al. [14] have quantified the PLC isoforms by the double-determinant tandem radioimmunoassay employing a set of monoclonal antibodies against each bovine brain isoform. They found in rat skeletal muscle crude extract 2 ng/mg of protein PLCβ (150–154 kDa), 30 ng/mg of protein PLCγ (145–148 kDa) and 30 ng/mg of protein PLCδ (85–88 kDa). The 110 kDa isoform described here as present in rabbit skeletal muscle was not detected in the rat system with the monoclonal antibodies employed. However, lack of cross-reactivity of the employed antibodies with the 110 kDa isoform cannot be excluded. During preparation of the rabbit muscle PLC different protease inhibitors were applied continuously; proteolytic degradation processes are unlikely but neither can they be excluded.

Assuming a purification factor of 30,000 for the PLC from rabbit fast skeletal muscle, a PLC concentration of ca. 30 ng/mg crude extract can be calculated. This value correlates with the concentration of PLCβ determined in several tissues [14].

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