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Interfacial hydrolysis of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate by turkey erythrocyte phospholipase C

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The activity of a β-isoform of phospholipase C (PLC) partially purified from turkey erythrocyte cytosol was assayed using phospholipid monolayers formed at an air–water interface. PLC was rapidly purified at least 8000-fold by a sequence of ion-exchange, hydrophobic and heparin chromatographies. ^{33}P-labelled substrates were prepared using partially purified PtdIns kinase and PtdIns4*P* 5-kinases, respectively, and purified by h.p.l.c. using an amino-cyano analytical column. Using such ^{33}P-labelled phosphoinositides of high specific radioactivity, PLC activity was monitored directly by measuring the loss of radioactivity from monolayers as a result of the release of inositol phosphates and their subsequent dissolution and quenching in the subphase. Under these conditions, PtdIns4*P* hydrolysis obeyed approximately first-order kinetics whereas PtdIns(4,5)P_2 hydrolysis was zero-order at least until 80 % of the substrate had been degraded. PLC activity was markedly affected by the surface pressure of the monolayer, with reduced activity at extremes of initial pressure and with the most permissive pressures in the middle of the range investigated. The optimum surface pressure for hydrolysis of PtdIns4*P* was approx. 25 mN/m, but for PtdIns(4,5)P_2 the maximum activity occurred at the markedly higher surface pressure of 30 mN/m. These data are discussed in terms of the substrate specificity and likely regulation of PLCβ isoforms engaged in degrading their substrate in biological membranes.

INTRODUCTION

Many cellular stimuli evoke the production of the second messengers Ins(1,4,5)P_3 and *sn*-1,2-diacylglycerol by activating members of the phospholipase C (PLC) family of phosphoinositide-specific phosphodiesterases [1–3]. Three major subfamilies of PLC have been identified, termed β, γ and δ, which differ in their respective modes of activation. Receptor stimulation of PLCβ subfamily members is mediated by α subunits of the Gq class of G proteins [4–6]. The relatively selective activation of PLCβ isoforms by G protein βγ subunits has also been described recently [7–10]. In contrast, PLCγ isoforms are activated by receptors with intrinsic tyrosine kinase activity, such as the epidermal growth factor and platelet-derived growth factor receptors [11,12] and also by receptors with associated tyrosine kinases, such as the T-cell receptor [13]. These catalyse the phosphorylation of tyrosine residues within the PLCγ sequence, leading to enhanced catalytic activity [14,15]. The factors regulating PLCδ are not known.

Although much is known concerning the activation and concomitant localization of PLC to the plasma membrane, relatively little has been reported concerning the interaction of PLCs with membrane substrates and the effects that membrane components such as non-substrate lipids may have on enzyme activity. The catalytic properties of lipases are characterized by pronounced increases in activity when substrate concentrations increase beyond the critical micelle concentration, causing aggregates of substrate lipid to form [16]. Several factors are thought to be responsible for increasing phospholipase catalytic activity at such aggregated lipid surfaces, including elevated local substrate concentration, favourable orientation of lipid molecules in the interface for catalysis, optimal lipid packing and distribution, favourable localized charge density and metal ion concentrations, and adoption of an active conformation by the enzyme upon penetrating the interface. Methods for analysing lipase catalytic activity *in vitro* have therefore been developed which involve aggregation of substrates, including immobilization on a solid support matrix [17], and formation of phospholipid emulsions [18]. One disadvantage of these approaches is that it is often impossible to vary the composition of the aggregate systematically and to influence physical properties such as the curvature of the interface and its surface pressure. Such problems can be overcome by the use of lipid monolayers at an air–water interface which can be manipulated so that the composition and physical properties mimic directly those of the inner leaflet of the plasma membrane where signalling events take place, culminating in the action of PLCs.

In a previous investigation of PtdIns-specific PLCs employing lipid monolayers, Hirasawa et al. [19] showed that the PtdIns-hydrolysing activity of a pig brain soluble extract was markedly affected by the monolayer surface pressure and that increasing acidic phospholipid content increased enzyme activity. More recently, Rebecchi et al. [20] reported that PLCδ activity against PtdIns(4,5)P_2 decreased 200-fold as monolayer surface pressure was increased from 20 to 40 mN/m. They also suggested that penetration of the monolayer involved less than 1 % of the PLC surface area, although the part of the molecule involved in interaction with the monolayer was not determined. Presumably, this interaction either would involve the putative catalytic X and Y domains [21,22] or would cause them to be located favourably for interaction with individual substrate molecules, permitting catalysis.

We have investigated degradation of phospholipid monolayers by the β-isoform of PLC isolated from turkey erythrocytes, with a view to obtaining a clearer understanding of its interactions with a membrane substrate. The development of this system has involved the purification of phosphoinositides from brain extract to sufficient homogeneity for these studies, the synthesis of high-

Abbreviations used: PLC, phosphatidylinositol-specific phospholipase C; Cho, choline; Etn, ethanolamine.

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specific-radioactivity ^{33}P-labelled phosphoinositides as substrates and the modification of existing purification protocols to allow the rapid partial purification of PLC from the cytosol of turkey erythrocytes. Our findings show that the catalytic activity of PLCβ can be monitored continuously by measuring the loss of radioactivity from a monolayer containing PtdIns4P or PtdInsP_2 labelled with ^{33}P. Increases in monolayer surface pressure resulted in a bell-shaped curve of PLC activity against both PtdIns$P$ and PtdInsP_2 and the data indicate that this form of PLC will hydrolyse PtdInsP_2 more rapidly than PtdIns$P$ at surface pressures approaching those of biological membranes.

MATERIALS AND METHODS

Materials

Dioleoyl phosphatidylcholine (PtdCho) and PtdIns (bovine brain) were obtained from Sigma. Dioleoyl phosphatidylserine (PtdSer) was synthesized by phospholipase D-catalysed head-group exchange on PtdCho as previously described [23]. [^{3}H]PtdIns$P$ and [^{33}P]ATP (cat. no. BF1000) were purchased from Amersham International. Type I Folch fraction from bovine brain was purchased from Sigma.

Partial purification of turkey erythrocyte cytosolic PLCβ

A method for the rapid purification of turkey erythrocyte PLC was developed, which has been used on five separate occasions with similar results. Packed erythrocytes were prepared from up to 12 litres of freshly collected citrated blood, which was washed three times with 1.5 mM Hepes (pH 7.4)/150 mM NaCl at 4 °C, yielding approx. 2.5 litres of packed cells.

Erythrocyte lysis

One litre of packed cells was diluted with 250 ml of Hepes saline and supplemented with 20 mM $MgCl_2$, 5 mM EGTA and 0.1 mM dithiothreitol plus a cocktail of protease inhibitors (0.1 mM phenylmethanesulphonyl fluoride, 0.1 mM benzamidine, and 1 mg/ml leupeptin, pepstatin, aprotinin). Cells were lysed in a Waring blender (low speed, 20–25 s) at 4 °C, which resulted in lysis of approx. 70 % of cells. Prolonged erythrocyte disruption was not used to avoid release of excessive quantities of DNA.

Ammonium sulphate precipitation

Cytosolic protein was precipitated using the method of Morris et al. [24], which avoids precipitation of haemoglobin. The resulting precipitate was separated from the haemoglobin-containing supernatant by centrifugation at 16000 g (10000 rev./min, JA-10 rotor, Beckman J2-21 centrifuge) for 20 min at 4 °C. The protein pellet was dissolved with homogenization in at least 20 vol. of 20 mM Tris/HCl, pH 8, 1 mM EDTA (buffer A) and the supernatant was clarified by centrifugation as above.

DE-52 anion-exchange chromatography

The clarified solution was diluted with 1.5 vol. of buffer A and batch loaded with gentle stirring on to 200 ml of DE-52 slurry, pre-equilibrated with buffer A, for 1 h at 4 °C. The resin was collected by filtration through a glass sinter, washed with 5 vol. of buffer A, and poured into a 2.5 cm diameter column, with a final bed height of approx. 60 cm. The column was developed with a 500 ml linear gradient of 0–0.5 M NaCl in buffer A and 10 ml fractions were collected. At this pH, PLC activity eluted between 100 and 125 mM NaCl, before the bulk of the protein. The resin was then washed with 1 M NaCl to clear all remaining protein.

Phenyl–Sepharose hydrophobic chromatography

Active fractions from DE-52 were pooled, NaCl content was increased to 700 mM, and loaded continuously on to a 60 ml phenyl–Sepharose column, pre-equilibrated with buffer A containing 700 mM NaCl. The column was washed with buffer A until the A_{280} of the eluate had returned to zero, which typically required approx. 100 ml. The column was developed with a 250 ml linear gradient of 0–50 % ethylene glycol in buffer A and 50 ml fractions were collected. The column was then unpacked and the resin was steeped in 100 ml of buffer A containing 60 % ethylene glycol, with stirring for at least 2 h at 4 °C. The resin was pelleted by centrifugation and PLC activity was assayed in all fractions.

Heparin–Sepharose chromatography

PLC from phenyl–Sepharose was loaded on to a 25 ml column of heparin–Sepharose pre-equilibrated with buffer A. The column was washed with buffer A until the A_{280} of the outflow was zero, and developed with a 200 ml linear gradient of 0–1 M NaCl in buffer A, containing 50 % (v/v) glycerol. Fractions of 5 ml were collected and PLC eluted at 450 mM NaCl. The extent of purification after this stage was never less than 8000-fold, and as much as 20000-fold, relative to the cytosol, with total protein reduced from > 200 g to < 5 mg. The presence of a 150 kDa PLC band in the purified protein was confirmed by immunoblotting using antiserum raised against denatured turkey erythrocyte PLC, the kind gift of Gary Waldo and T. K. Harden (University of North Carolina).

Table 1 details one of the purifications achieved using this method. The protocol was devised to permit rapid purification of PLC to maximize yields of PLC, which was achieved by elimination of prolonged steps such as dialysis for the removal of salt. In our hands, the purified PLC activity required 50 % glycerol for stability, but storage at −70 °C under these conditions resulted in no detectable loss of activity over at least 3 months.

Micellar PLC assay

Mixed phospholipid vesicles of PtdSer/PtdEtn/[^{3}H]PtdIns$P$ (Etn, ethanolamine) (1:1:1, by molar proportion) were prepared by sonication of dried lipid films into 10 mM Hepes, pH 7.4, to give a final concentration of 200 μM PtdIns$P$. Assays (100 μl final volume) contained 25 μl of 2 % sodium cholate, 25 μl of buffer B (10 mM Hepes, pH 7.4, 480 mM KCl, 40 mM NaCl, 8 mM EGTA, 23.2 mM $MgCl_2$, 8.4 mM $CaCl_2$), and 25 μl of protein fraction, to which was added 25 μl of lipid substrate to start the reaction, as previously described [24]. Reactions were terminated by addition of chloroform/methanol/HCl (40:80:1, by vol.) and separation of phases was achieved by sequential addition of H_2O, chloroform and 0.1 M HCl, with centrifugation. InsP_2 production was determined in 500 μl of the upper phase by liquid scintillation spectrometry.

Partial purification of human erythrocyte PtdIns kinase

PtdIns kinase was partially purified according to the methods of MacPhee et al. [25] and Graziani et al. [26] from human erythrocyte ghosts prepared from 200 ml of whole blood. The partial purification procedure increased the enzyme specific activity approx. 5-fold to 330 pmol/min per mg and enzyme aliquots were stored at −20 °C until used. PtdIns kinase was partially purified in this manner to obtain a form of the enzyme that was free of membrane phospholipids including PtdIns,

Table 1 Partial purification of PLC from turkey erythrocyte cytosol

PLC was purified from 2 litres of blood as described in the Materials and methods section. Enzyme activity was determined in all fractions using PtdIns*P* concentrations of 5 times the quoted K_m (approx. 8 μM) [24]. Protein was determined by haemoglobin colorimetric assay (cytosol) or by the method of Bradford [42].

Fraction	Volume (ml)	Protein (mg)	Activity (nmol/min)	Specific activity (nmol/min per mg)	Yield (%)
Cytosol	200	40200	155.4	0.0039	100
Clarified supernatant	400	4100	302.6	0.074	> 100*
DE52	50	115	62.7	0.542	40.4
Phenyl–Sepharose	45	10.5	56.7	5.5	36.5
Heparin–Sepharose	24	0.96	22.8	23.9	14.7

* The artificially elevated yield (based on total PLC activity recorded) was possibly due to proteolytic PLC fragments with amplified activity or release of the purified enzyme from some form of inhibition, but was not routinely observed.

which if retained would reduce the specific radioactivity of the radiolabelled PtdIns*P* required in these assays.

PtdIns kinase assay

PtdIns (450 μM) was sonicated into 1.2% (v/v) Triton X-100, using a probe-type sonicator, to which was added an equal volume of 200 μM Hepes, pH 7.4, 40 mM $MgCl_2$, 4 mM EGTA, 4 mM dithiothreitol, 400 mM NaCl (buffer C). [^{32}P]ATP (50 μM) was added, and then 50 μl of this substrate cocktail was added to 50 μl of column fraction at 37 °C. The reactions were terminated after 10 min as described for the PLC assay. However, after centrifugation, the upper aqueous phase was aspirated to waste and the lower phase was washed three times with synthetic upper phase. The lower phase was then transferred to a clean tube, and radioactivity was detected by Cerenkov counting. The increase in ^{32}P in the lower phase was taken as a measure of PtdIns kinase activity, and the presence of [^{32}P]PtdIns*P* was confirmed by t.l.c. using a solvent of chloroform/methanol/water/NH_3 (100:75:25:15, by vol.).

Partial purification of rat brain PtdIns*P* kinase

Cytosolic PtdIns*P* kinase was partially purified from 10 rat brains according to the method of Ling et al. [27], and stored at −20 °C until used. PtdIns*P* kinase was purified to improve the yields of ^{33}P-labelled PtdInsP_2 (see below) by removal of contaminating phosphomonoesterases and PLCs.

PtdIns*P* kinase assay

PtdIns*P* (5 μM) was sonicated into 50 mM Tris/acetate, pH 7.4, 80 mM KCl, 10 mM magnesium acetate, 2 mM EGTA (buffer D). A 20 μl sample of column fraction was added to 80 μl of PtdIns*P* suspension and the reaction (37 °C, 10 min) was started by the addition of 50 μM [^{32}P]ATP in buffer D. Termination and product analysis were performed as for the PtdIns kinase assays.

Preparation of [^{33}P]PtdIns*P* and [^{33}P]PtdInsP_2

[^{33}P]PtdIns*P* and [^{33}P]PtdInsP_2 were prepared using partially purified PtdIns kinase and PtdIns*P* kinase as described above, with the following modifications. PtdIns (400 of nmol) was sonicated into 250 μl of 1.2% Tween 20, to which was added 250 μl of buffer E, giving 800 μM PtdIns. [^{33}P]ATP (10 μl) (specific radioactivity > 1000 Ci/mmol) was added to this substrate suspension with no unlabelled ATP. This protocol was adopted to ensure that lipid of high specific radioactivity was produced. Assays were performed in 100 μl volumes, with 50 μl of enzyme solution being added to 50 μl of substrate. Tween 20 was used because of its high hydrophile/lipophile ratio, which ensured that it would wash out into the upper phase after the reaction and not contaminate the lipid phase. Preparations of [^{33}P]PtdIns*P* in which Triton X-100 was used in the enzyme mixture proved unsuitable for monolayer studies because of the presence of surface-active contaminants (presumably Triton X-100). PtdIns kinase assays therefore contained only very low concentrations of Triton X-100 derived from the enzyme preparations. The assay, performed for 3 h with occasional mixing at 37 °C, yielded approx. 3% incorporation of the γ-phosphate of ATP into PtdIns*P*.

For PtdIns*P* kinase, the PtdIns*P* concentration was 50 μM and no unlabelled ATP was included. Similar incorporation of the γ-phosphate of ATP into lipid was achieved. Radiolabelled PtdIns*P* and PtdInsP_2 prepared by these procedures were purified by h.p.l.c. as described in the Results section.

Preparation of unlabelled PtdIns*P* and PtdInsP_2

PtdIns*P* and PtdInsP_2 were purified from type I Folch fraction from bovine brain, based on the method of Low [28]. Phosphoinositides were repeatedly methanol-precipitated from 1 g of lipid, dissolved in solvent A (chloroform/methanol/water, 20:9:1, by vol.) and applied to a 60 ml DEAE-cellulose column pre-equilibrated with solvent A. The column was washed with 2 vol. of solvent A and the resultant eluate contained some PtdSer and PtdIns. The column was developed with a 300 ml linear gradient of 0–0.3 M ammonium acetate in solvent A and 10 ml fractions were collected into acetone- and chloroform-washed borosilicate glass tubes. In contrast with the results of Low [28], in our hands, PtdIns*P* eluted at 150 mM ammonium acetate and PtdInsP_2 eluted at 200 mM ammonium acetate. Residual PtdIns and PtdSer eluted from the column at 0–50 mM ammonium acetate. This purification has been repeated numerous times, with identical results. Because of some tailing of PtdIns*P*, pooled lipid samples were washed with 2 M NaCl in methanol and re-chromatographed on DEAE-cellulose twice. All vessels and tubes with which the lipid came into contact were kept scrupulously clean by rinsing with distilled water, acetone and chloroform. This was to ensure that no residual surface-active contaminants remained in the lipid preparations. This protocol resulted in lipids of single species, by t.l.c. analysis, yielding > 7 μmol of PtdIns*P* and > 15 μmol of PtdInsP_2. Lipids were cleaned as above, dried to a film under nitrogen and redissolved in 5 ml of chloroform, 0.7 ml of methanol and 50 μl of water. Lipids were stored under nitrogen at −70 °C.

Monolayer methodology

All enzyme experiments were performed using a 19 ml volume square trough of surface area 29.61 cm^2, milled out of Teflon, which contained an injection port separated from the lipid monolayer; this permitted access to the subphase without perturbation of the lipid film. The monolayer surface pressure was monitored continuously throughout the assay, using a Wilhelmy plate attached to an electromicrobalance, as previously described [29]. The subphase was stirred using a Teflon-coated magnetic stirrer bar, which was seated in a small well beneath the subphase. The apparatus was contained within a large cabinet which permitted thermostatic regulation, and all assays were performed at 30 °C. In between assays, the trough was washed sequentially with tap water, brushed with detergent, rinsed with tap water, wiped with ethanol and rinsed twice with distilled water and dried by aspiration.

Monolayers were formed from a mixture comprising 70% PtdCho, 27% PtdSer and 3% PtdIns$P$ or PtdInsP_2 (by molar proportion) in chloroform solvent, containing radiolabelled substrate. Lipids were applied with a Hamilton syringe to the surface of an aqueous subphase (composed of 20 mM Hepes, pH 7.4, 120 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA) which had previously been swept clean of all other surface-active material using a Teflon bar. Pressure was monitored as described, and 0.7 ml of subphase was replaced with 0.7 ml of enzyme preparation (20–30 μg of protein) only after a stable monolayer had formed whose pressure was constant. This protocol maintained the volume of the subphase at 19 ml. After 5 min, Ca^{2+} ions were added to the subphase to the desired concentration to start catalysis and either the reaction was monitored continuously (^{33}P-labelled monolayer) or 0.5 ml aliquots were taken at several time points (^{3}H-labelled lipids). For continuous recordings of PLC activity, using ^{33}P-labelled lipids, radioactivity in the monolayer was detected using a methane/argon gas-flow radio detector suspended approx. 0.5 cm above the lipid film. Enzyme-mediated catalysis resulted in the loss of radioactivity from the monolayer in the form of InsP_2/InsP_3, which dissolved in the subphase and was thereby quenched. ^{33}P was used in preference to ^{32}P because similar high specific radioactive labelling of the lipids was possible, with the added advantage that the radioactive energy is approx. 7-fold less, resulting in a higher degree of quenching by the subphase and hence improving the signal-to-noise ratio. The extent of lipid hydrolysis was determined at the end of catalysis by sampling the radioactivity remaining in the monolayer and in an aliquot of the subphase.

Surface pressure/area isotherms for purified inositol phospholipids and PLC substrate cocktails were measured at the air–water interface at 22 °C using a Teflon trough (32.2 cm × 17.3 cm) which was placed in a thermostatted chamber. The subphase contained 10 mM Tris/HCl, pH 7.4. Lipid (25 or 50 nmol) was spread and compressed using a motorized Teflon barrier at a rate of 86.5 cm^2/min. Monolayer surface pressure and surface area were monitored continuously throughout the compression.

RESULTS

Partial purification of PLC

The chromatographic purification of PLC (see the Materials and methods section) was designed to give a rapid partial purification which excluded prolonged steps such as dialysis between subsequent columns. Elution of PLC from DE52 resin with salt meant that reverse-phase chromatography would be the most appropriate second column. All the phosphoinositide-hydrolysing activity from the DE52 column bound tightly to phenyl-Sepharose resin, and eluted only in the 60% (v/v) ethylene glycol wash of the resin after the column was unpacked. Up to 70% of the PLC activity was recovered from phenyl-Sepharose, and this chromatography consistently produced a 10-fold purification of the enzyme. A typical elution profile of protein and PLC activity is shown in Figure 1.

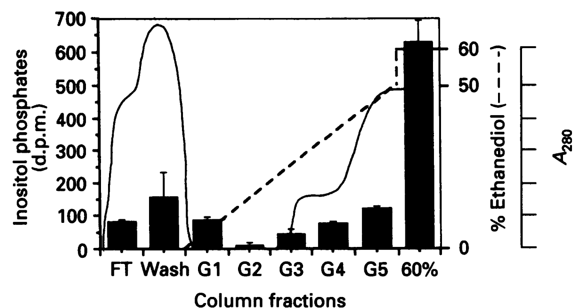

Figure 1 Phenyl–Sepharose chromatography of PLC

Phosphoinositide-hydrolysing fractions from DE52 were pooled, the salt concentration was increased to 0.7 M and loaded continuously on to a phenyl-Sepharose column as described in the Materials and methods section. The major PLC activity eluted with 60% (v/v) ethanediol. Bars are mean ± range of duplicate determinations of PLC activity in each fraction. FT, column flow through; wash, no salt wash; G1–G5, fractions 1–5 of the ethanediol gradient; 60%, 60% ethanediol wash of the resin. The active fraction was further chromatographed using heparin–Sepharose.

Phenyl-Sepharose has been used previously for the purification of PLC activities from brain [30] and comparison of the elution profiles shows that turkey erythrocyte PLCβ behaves differently from at least four other brain PLC activities on this resin. The interaction of turkey erythrocyte PLC with phenyl-Sepharose was apparently much stronger than for any of the brain activities, and required longer incubation with high concentrations of non-polar solvent for release from the resin, as stated above. This strong interaction of PLC with the hydrophobic resin is probably the cause of the relatively high degree of purification of the enzyme through this step.

^{33}P-labelled phosphoinositides

Synthesis of ^{33}P-labelled phosphoinositides yielded lipids of high specific radioactivity (> 1000 Ci/mmol) such that their inclusion in phospholipid monolayers contributed no significant surface activity to the cocktail. Monolayers typically contained 10000–30000 d.p.m. of ^{33}P. The noise in the signal gave a fluctuation of 18.8 ± 5.1% about the mean signal in the higher sensitivity mode and 12.4 ± 2.6% in the lower sensitivity mode. Thus a clear signal could be detected using ^{33}P-labelled monolayers, and PLC-catalysed reductions in radioactivity could be readily measured as inositol phosphates were released into the subphase and the radioactivity was quenched (see below). In principle, ^{14}C-labelled phosphoinositides would possess appropriate quench characteristics for use in such assays, but the specific radioactivities obtained in pilot experiments were insufficient to provide signals of sufficient strength for direct measurement of PLC activity and would have required the use of monolayers containing a relatively high proportion of the polyphosphoinositide substrate. Because of its high energy, ^{32}P would be expected to give a poorer signal-to-noise ratio than ^{33}P, and we believe that ^{33}P is therefore the most appropriate radionuclide for these assays.

The use of Tween 20 in preparative PtdIns kinase assays for

the synthesis of [^{33}P]PtdIns$P$ rather than the more lipophilic Triton X-100, and the subsequent purification of both PtdIns$P$ and PtdInsP_2 by h.p.l.c., eliminated all surface-active contaminants from the lipids. ^{33}P-labelled lipids were purified by h.p.l.c. using an amino-cyano analytical column (Laserchrom Analytical Ltd.) pre-equilibrated in solvent A (described in the Materials and methods section). Baseline resolution of PtdIns, PtdIns$P$ and PtdInsP_2 was obtained routinely using the elution procedure described below, representing a substantial improvement on a similar, previously published method [28]. The improved resolution is probably attributable to the presence of an additional interactive group (the cyano moiety) in the column matrix that we employed.

The column was developed with the following protocol: 0 min, 0 % solvent B (solvent A containing 0.6 M ammonium acetate); 10 min, 0 % B; 60 min, 100 % B; 100 min, 100 % B; 101 min, 0 % B; and washed with solvent A for at least 30 min. Unlabelled PtdIns substrate eluted from this column at approx. 50 mM salt (retention time of 13 min), PtdIns$P$ eluted at 0.4 M and PtdInsP_2 at 0.51 M salt. Recovery off the column was stoichiometric. Lipids were desalted by repeated phase splits comprising 0.5 vol. of methanol and 0.5 vol. of acidified 2 M NaCl. Recovery of lipids after three phase splits was 76 ± 2 %. A typical elution profile is shown in Figure 2. The figure shows the separation of ^{3}H-labelled phosphoinositides for ease of comparison of the behaviour of all three lipids.

The generation of homogeneous lipids is crucial to the production of reproducible data in this monolayer system. Initial studies employing ^{33}P-labelled substrates which contained trace amounts of some non-lipid contaminant with a collapse pressure of approx. 33 mN/m (presumably detergent) did not show any surface pressure–activity relationship for PLC (S. R. James, R. A. Demel and C. P. Downes, unpublished work). Thus, the contaminant apparently abrogated the effects of monolayer surface pressure on PLC activity towards the substrate ^{33}PtdIns$P$, presumably by facilitating enzyme penetration into the monolayer, which resulted in > 80 % hydrolysis at all pressures between 20 and 35 mN/m.

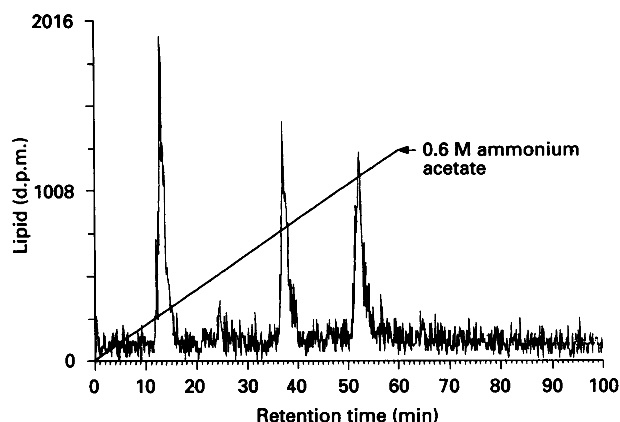

Figure 2 Amino-cyano h.p.l.c. of phosphoinositides

[^{3}H]PtdIns, [^{3}H]PtdIns$P$ and [^{3}H]PtdInsP_2 were loaded in approx. equal amounts on to an amino-cyano analytical column (Laserchrom Analytical) and eluted using a 0–0.6 M ammonium acetate linear gradient, as described in the Results section. The major peaks are, from left to right, PtdIns, PtdIns$P$ and PtdInsP_2 respectively. Radioactivity was determined using an 'in-line' radiodetector ('Radiomatic'; Packard).

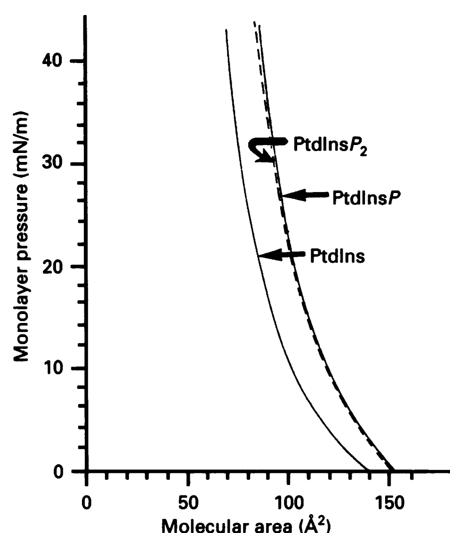

Figure 3 Pressure–area isotherms for purified phosphoinositides

Monolayers containing inositol phospholipids of known mass were formed over a monolayer trough of surface area 350 cm^2. Lipid films were compressed at a constant rate and surface pressure and average molecular area were determined continuously. Traces are representative of at least two separate determinations and show molecular areas for surface pressures up to 40 mN/m. Lipid films collapsed at a rate of approx. 43 mN/m.

Phospholipid molecular areas

The molecular areas of the PtdCho/PtdSer/phosphoinositide lipid mixtures and of inositol phospholipids purified by h.p.l.c. as described above were determined for all of the pressures used in this study. Monolayers of a given mass of lipid were formed over a monolayer trough and compressed continuously with simultaneous measurement of the lipid molecular area. Figure 3 shows compression isotherms for the purified phosphoinositides, plotted as molecular area against monolayer surface pressure. The data demonstrate that whereas PtdIns$P$ and PtdInsP_2 barely differ from each other in their behaviour in monolayers, both exhibit significantly expanded molecular areas relative to PtdIns at all pressures. The increased charge in the inositol headgroup of the polyphosphoinositides therefore appears to increase steric effects between adjacent lipid molecules, thereby increasing individual molecular areas. Lipid mixtures used in monolayer PLC assays contained only 3 % (by molar proportion) phosphoinositide and the average molecular area of these cocktails was dictated by the predominant lipid constituent, PtdCho. The pressure–area isotherm for the lipid cocktail was similar to that reported by Rebecchi et al. [20] (results not shown). The molecular area of PtdCho was 65–62 Å^2 within the pressure range 30–35 mN/m, which is thought to be the area of PtdCho in bilayers [31], indicating that physiologically relevant monolayer pressures lie within this range.

Interfacial phosphoinositide hydrolysis by PLC

In all monolayer assays, before addition of enzyme, approx. 2–4 % of the monolayer label immediately dissolved in the subphase. No further loss of radioactivity was seen until after the addition of PLC. Hydrolysis of PtdIns$P$-containing monolayers was absolutely dependent on the Ca^{2+} concentration of the subphase and all data presented here were obtained using 10 μM Ca^{2+}, which sustains the maximum rate of PtdIns$P$ hydrolysis. The rate of catalysis was significantly slower in a dose-dependent fashion at all Ca^{2+} concentrations below 10 μM, and in the absence of any added Ca^{2+} (free [Ca^{2+}] $\approx 10^{-8}$ M), no loss of

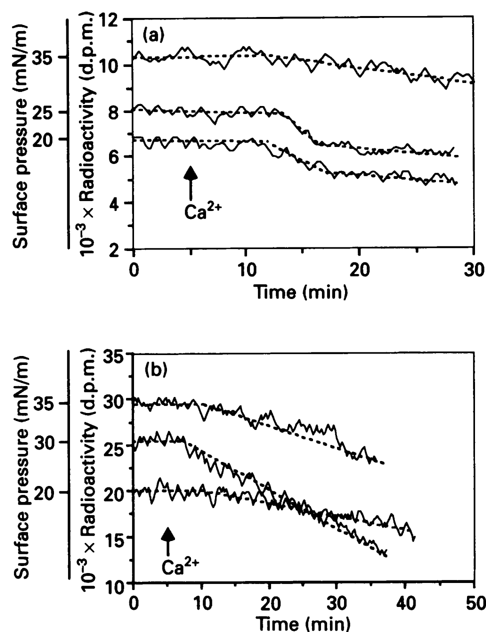

Figure 4 Continuous trace recordings of PLC-catalysed loss of radioactivity from ^{33}P-labelled phospholipid monolayers

Monolayers were formed at different initial surface pressures and PLC was added to the subphase after the monolayer had stabilized, at time called zero. Ca^{2+} ions to give a free concentration of 10 μM were added after 5 min and the reactions were allowed to proceed for a further 20 or 30 min. Traces are representative of at least three experiments at each pressure. (**a**) PtdIns*P*-containing monolayers; (**b**) PtdIns*P*$_2$-containing monolayers.

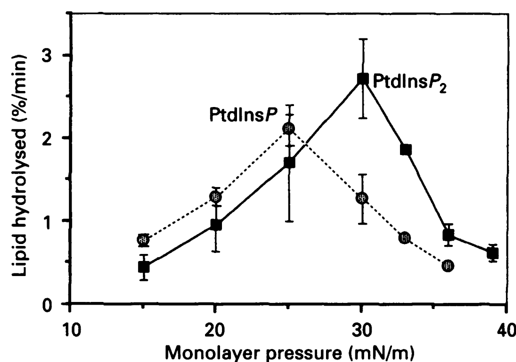

Figure 5 Surface pressure–PLC activity relationships for both PtdIns*P*- and PtdIns*P*$_2$-containing monolayers

PLC activity was determined against monolayers formed at increasing initial surface pressures. Reaction times were 20 or 30 min after addition of Ca^{2+} and data are expressed as percentage of lipid hydrolysed per min. Data are means ± S.D. of at least three experiments for each pressure.

radioactivity from PtdIns*P*-containing monolayers was observed, as illustrated in traces recorded before Ca^{2+} addition (see Figure 4a). Hydrolysis of PtdIns*P*$_2$-containing monolayers was also accelerated with increasing concentrations of Ca^{2+}, but PLC activity was not absolutely dependent on added Ca^{2+} for this substrate. A detailed examination of the Ca^{2+} dependency of this isoform of PLC will be presented elsewhere. As for PtdIns*P*, PtdIns*P*$_2$ hydrolysis was analysed using 10 μM Ca^{2+} in the subphase, a concentration which supports maximum catalytic rate.

Typical continuous trace recordings of the changes in monolayer radioactivity upon addition of PLC and Ca^{2+} are shown for PtdIns*P* (Figure 4a) and PtdIns*P*$_2$ (Figure 4b). Variations of the initial surface pressure of the monolayer resulted in markedly different rates of loss of radioactivity into the subphase. Catalytic activity against PtdIns*P* was biphasic, featuring an initial fast rate which declined progressively with time. This activity was preceded by a pronounced lag time between addition of Ca^{2+} ions and the onset of catalysis. In contrast, the activity of turkey erythrocyte PLC against PtdIns*P*$_2$-containing monolayers was not preceded by a marked lag phase and proceeded at a uniform rate. No asymptotic phase was reached at any pressure, even after 80 % of the substrate had been degraded. A detailed analysis of lag times for pig brain extract PLC activity towards PtdIns has been reported previously by Hirasawa et al. [19] and was rationalized as the time required for enzyme penetration into the monolayer and adoption of an active conformation. The data presented here suggest that the lag time may be determined by other factors in addition to enzyme penetration, such as the suitability of the lipid as a substrate for PLC and monolayer surface quality. The kinetics of the maximum rate of hydrolysis of PtdIns*P*-containing monolayers (observed at 25 mN/m; Figure 4a) was not significantly different from the greatest rate for PtdIns*P*$_2$, seen at a surface pressure of 30 mN/m (9.5 ± 1.9 pmol/min compared with 8.0 ± 1.3 pmol/min for PtdIns*P* and PtdIns*P*$_2$ respectively). Indeed, at all monolayer pressures up to 25 mN/m, the initial fast phase of hydrolysis of PtdIns*P* was not significantly different from that of PtdIns*P*$_2$, and only at 30 mN/m and above was the rate of PtdIns*P*$_2$ degradation greater than that of PtdIns*P* at the same pressures.

To facilitate a comparison of PtdIns*P* hydrolysis with PtdIns*P*$_2$ degradation, Figure 5 shows data transformed into the percentage of lipid hydrolysed per min, disregarding the biphasic nature of the kinetics for PtdIns*P* and expressed against the initial surface pressure of the monolayer. Experiments were performed over a 30 min period, at the end of which the slow phase of PtdIns*P* hydrolysis was well advanced. Radioactivities remaining in the monolayer and present in the subphase were determined after this time and catalysis was expressed as lipid hydrolysed per unit time. Expressing the results in this way has the effect of averaging out hydrolysis of PtdIns*P* over the whole incubation period while allowing direct comparison of PtdIns*P* degradation with PtdIns*P*$_2$ hydrolysis. In the early portion of the pressure–activity curve, PLC activity increases as surface pressure increases (Figure 5). The curve is bell-shaped, however, and a peak in PLC activity for both PtdIns*P* and PtdIns*P*$_2$ is seen; as pressure increases beyond this point, PLC activity is markedly reduced. The most permissive surface pressures for PtdIns*P* and PtdIns*P*$_2$ are 25 mN/m and 30 mN/m, respectively. Figure 5 emphasizes the fact that within the pressure range 30–36 mN/m, PLC activity towards PtdIns*P*$_2$ is greater than that towards PtdIns*P*.

DISCUSSION

Turkey erythrocytes are a relatively uncomplicated cell type in which activation of the phosphoinositide cycle has been well defined. Thus the endogenous PLC, some of which is cytoskeleton-associated [32], is activated by purinergic and β-adrenergic agonists via distinct populations of receptors [33,34], mediated by an avian homologue of G_{11} (S. R. James, C. Vaziri, G. Milligan and C. P. Downes, unpublished work) [37]. The cytosolic PLC isoform used in these studies, which forms > 90 % of the phosphoinositide-hydrolysing activity of turkey erythrocytes, can also be stimulated by high concentrations of βγ subunits of G proteins [8], and is therefore more similar to

mammalian PLCβ2 than PLCβ. To define further the regulation and catalytic activity of this PLC isoform, we have undertaken studies investigating the interfacial behaviour of this enzyme using substrates in phospholipid monolayers.

It is not known whether the simple redistribution of PLC to close juxtaposition with its substrate is the most important factor in switching on catalytic activity or whether the enzyme must adopt an active conformation before phosphoinositide hydrolysis can occur. By analogy with other lipases [35,36], the latter idea seems a likely possibility, and penetration of the enzyme into its membrane substrate may promote the changes required in enzyme conformation for catalysis. An alternative to the mechanism proposed above is that PLCs might be restrained in intact cells by their association with inhibitory proteins [3]. However, PLCβ isoforms, including the turkey erythrocyte enzyme, can be substantially activated in reconstitution experiments containing purified enzyme and G protein subunits [37]. Although these observations do not preclude the existence of inhibitory proteins, they are not necessary to explain the activation of PLCβ isoforms by $G_{\alpha/11}$-coupled receptor systems. As discussed below, our results suggest that the physico-chemical properties of the membrane suffice to limit PLC activity at relevant surface pressures. This may explain why variations in the composition of phospholipid vesicle substrates affect the basal rate of PLC activity and hence their capacity to be activated in the presence of G proteins [38].

The cytoskeletal localization of G protein-regulated PLC in turkey erythrocytes is an arrangement that probably overcomes any diffusional constraints that would apply if PLC were recruited from the cytosol. This arrangement probably does not directly influence the type of interaction that must take place between a soluble phospholipase and its substrate in a membrane. Monomolecular phospholipid films can be used as a well-defined membrane model in which to study the interactions of PLCs with membrane-like substrates, as opposed to the more commonly-used mixed micelle and liposome assays, and we believe they may be useful in addressing some of the issues described above.

The data presented here show that, using ^{33}P-labelled substrates to measure PLC activity directly, the rates and extents of Ptd*Ins*P and PtdInsP_2 hydrolysis in a PtdCho/PtdSer composite monolayer differ from each other and are surface pressure-dependent. Although increasing surface pressure increases phosphoinositide mass in the monolayer, this was not accompanied by a simple increase in enzyme activity throughout the pressure range investigated. The reduction in PLC activity as initial monolayer pressures were increased above the most permissive pressures was presumably the result of a diminution in the ability of the enzyme to penetrate the substrate. This conclusion is based on the fact that monolayer assays of 20 or 30 min were accompanied by significant increases in surface pressure, possibly resulting from the interaction of other proteins with the monolayer in addition to PLC. However, there was no pressure-induced change in the rate of catalysis during the course of the experiments (see Figure 4), which indicates that it is the initial surface pressure that is crucial in determining the subsequent penetration of PLC and rate of PLC activity. The lower rate of catalysis of lipids at lower initial surface pressures, which would be expected to permit relatively easy penetration by PLC, is possibly due to enzyme denaturation by unfolding at the monolayer.

The monolayer composition in these assays (70% PtdCho, 27% PtdSer and 3% phosphoinositide, by molar proportion) was chosen as a simplified model of the membranes with which PLC is likely to interact. There are clear precedents, however, which suggest that PLC activity is profoundly affected by the presence of other components in the monolayer. Thus, Hirasawa et al. [19] showed that increasing phosphatidic acid content at the interface permits PtdIns hydrolysis at surface pressures that are otherwise non-permissive. In addition, we have previously shown a different pressure–activity relationship for PLC against PtdIns$P$ in this monolayer system [39]. The curve generated showed peak activity at a surface pressure of 15 mN/m, no differences in PtdIns$P$ degradation at 20–33 mN/m, and a cut-off of activity at higher pressures, an effect attributable to the PtdSer content of the monolayer (results not shown). These conditions, which can be manipulated systematically in the monolayer assay system, will be analysed in detail in further work.

Furthermore, PLC activity may be affected by the composition of the subphase in these assays. The subphase buffer was designed to be a simplified intracellular-type buffered salt solution and comprised largely KCl (120 mM) with 20 mM NaCl contributed by the enzyme preparation. The choice of composition of the subphase buffer is important because it influences various aspects of the monolayer system. The resultant free Ca^{2+} concentration is determined in some part by the ionic strength of the solution, and the ionic composition would be expected to alter the charge characteristics at the phospholipid monolayer, and may influence consequent PLC activity. PLC activity would therefore be predicted to be influenced by several characteristics of the monolayer system, such as the Ca^{2+} concentration, the charge located at the interface and the phospholipid composition of the interface. The activity of turkey erythrocyte PLCβ was markedly influenced by the free [Ca^{2+}] and the enzyme was able to hydrolyse 100% PtdIns$P$ monolayers but not 100% PtdInsP_2 monolayers (S. R. James, R. A. Demel and C. P. Downes, unpublished work), an effect presumably attributable to the larger negative charge at the PtdInsP_2 monolayer. Further work on these aspects of PLC interfacial hydrolysis of phosphoinositides is required for a more complete understanding of the factors that regulate PLC activity in monolayer systems.

Initial studies of phosphoinositide signalling in turkey erythrocytes showed that stimulation of 'ghost' preparations with non-hydrolysable analogues of GTP caused the rapid hydrolysis of PtdInsP_2, whereas PtdIns$P$ was a relatively minor substrate and PtdIns, present in much larger amounts than other inositol phospholipids, was not hydrolysed at all [40,41]. In contrast, assays of purified turkey erythrocyte PLC, using cholate/substrate mixed micelles, showed that PtdIns$P$ and PtdInsP_2 were equivalent substrates (F. Ruiz-Larrea and C. P. Downes, unpublished work) and that PtdIns could be hydrolysed, but at a substantially slower rate. In this study, we have shown that the physicochemical properties of monolayers, such as initial surface pressure, affect both the rate of hydrolysis and substrate specificity. Thus, PtdIns$P$ and PtdInsP_2 were equally effective substrates at low surface pressures, but PtdInsP_2 was preferred at monolayer surface pressures at or approaching those experienced in cell membranes. It would be interesting to establish pressure–activity curves using PtdIns as a substrate for this class of PLC; we predict that PtdIns should be a very poor substrate at high surface pressures. The pressure–activity relationship for PLC activity against PtdInsP_2-containing monolayers presented here contrasts with that previously reported for PLCδ [20]. The specific activity of this isoform of PLC was shown to decrease linearly with increasing monolayer surface pressure, with maximum activity being observed at the lowest pressures investigated (15 mN/m). The basis for the differences between PLCβ and PLCδ activity in monolayers is not clear but it establishes the interesting phenomenon that different isoforms of the same family of enzymes are affected differently by the quality of the interfaces with which they interact.

In summary, we have described the establishment of a finely

controlled monolayer system for studying the family of PLCs, which will permit investigations into many aspects of the interaction of the PLCs with their substrates and their regulation. Using this system, we have shown that PLC activity towards both PtdIns*P*- and PtdInsP_2-containing monolayers is surface pressure-dependent, and that at pressures approaching those of membrane structures, PtdInsP_2 hydrolysis is favoured over PtdIns*P* hydrolysis.

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