Role of lipid packing in the activity of phospholipase C- δ_1 as determined by hydrostatic pressure measurements

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Previous studies with phospholipid monolayers revealed a large decrease in the activity of phosphoinositide-specific phospholipase C- δ_1 (PLC- δ_1) which catalyses the hydrolysis of PtdIns(4,5) P_2 as lateral pressure is applied to the membrane. If stress on the membrane is the sole inhibitor of PLC- δ_1 activity, the enzyme must penetrate the membrane surface to engage its substrate. To test the effect on PLC- δ_1 activity of lipid packing in the absence of a directional stress, we examined the effects of increasing hydrostatic pressure on enzymic activity. We find that, in contrast with monolayer studies, increasing lipid packing by hydrostatic pressure does not affect membrane binding and

increases enzymic activity by 90 % in going from atmospheric pressure to 10⁸ Pa (approx. 1000 atm). The increase in activity could be accounted for mainly by electrostriction of water around the multiply-charged product. Our results show that when there is no net stress on the monolayer, lipid packing does not alter PLC- δ_1 activity, possibly because penetration of the enzyme into the membrane surface is shallow. We suggest that, in biological membranes, the activity of this and possibly other interfacial proteins is independent of headgroup packing.

Key words: catalysis, fluorescence, phosphatidylinositol.

INTRODUCTION

Phosphoinositide-specific phospholipase C- δ (PLC- δ) catalyses the hydrolysis of the lipid PtdIns(4,5) P_2 , producing the second messengers Ins(1,4,5) P_3 and diacylglycerol, which cause the release of Ca²⁺ from intracellular stores and the activation of protein kinase C (PKC) respectively (reviewed in [1,2]). Because PLC is an interfacial enzyme it is not surprising that its enzymic activity is affected by the properties of the membrane such as headgroup charge, hydration, structure and packing [3]. This last property, lipid packing, presumably regulates PLC- δ activity by restricting access to substrate. Several studies have shown that the activity of PLC- δ when its PtdIns(4,5) P_2 substrate is incorporated into membrane bilayers is 2–10 % of that towards detergent micelles, in which access to substrate is essentially unrestricted [4]. In phospholipid monolayers, lateral pressures of 20–40 mN/m decrease the catalytic rate by two orders of magnitude, suggesting a simple model in which a portion of the enzyme of approx. 1 nm² must insert into the membrane surface [5]. PLC- β and PLC- γ isoenzymes are also inhibited by increased lateral pressure, although they exhibit a steeper dependence over a much narrower range, becoming independent of surface pressures above 30–35 mN/m [5,6].

To improve our understanding of the ability of lipid packing to affect PLC- δ_1 activity, we have measured its catalytic rate under elevated hydrostatic pressure to complement previous monolayer studies. Hydrostatic pressure allows us to observe the effect of packing on PLC- δ_1 with the use of lipid bilayers, which is a more appropriate model system than monolayers. Hydrostatic pressure uniformly compresses the protein and membrane bilayer, and its effect on lipid packing is directly opposite to that of temperature [7]. In contrast, lateral pressure, used in monolayer studies, exerts an upward force on proteins that have inserted into the surface, causing them to be eliminated from the membrane.

High pressure has been useful in the study of protein systems [7]. Pressure is often used to study protein folding because increasing the pressure forces aqueous solvent into the protein matrix to fill in any void volumes. Although only small conformational changes are seen from 1 atm to approx. 500 MPa (1 atm ≈ 100 kPa), higher pressures might denature proteins. The application of pressure to an enzyme-catalysed reaction will either increase or decrease the reaction rate depending on the sign of the activation volume, ΔV^* . Of the approx. 50 enzymes whose catalytic rates have been studied under pressure, a few are activated by pressure but most have a positive ΔV^* in the range 20–40 ml/mol [8]. The effect of pressure on ΔV^* is thought to be a result of a stabilizing of a particular conformational state of the protein, although other factors such as the promotion of electron tunnelling during a catalytic step might also be involved. Although the effect of hydrostatic pressure on aqueous soluble and integral membrane proteins has been investigated, its effect on an interfacial lipolytic enzyme operating on a membrane bilayer has not previously been determined.

In the present study we measured the effects of pressure on the membrane binding and enzymic activity of PLC- δ_1 . To conduct activity measurements under pressure, we developed a simple spectroscopic assay that allowed us to view the activity by fluorescence. Our studies indicate that lipid packing in fluid biological membranes does not significantly influence access to substrate.

EXPERIMENTAL

The expression of human recombinant His₆-PLC- δ_1 in *Escherichia coli* has been described [9]. Before each experiment, the protein was dialysed overnight against 150 mM Chelex-treated NaCl (made by passing NaCl twice over a 200 ml bed of Chelex-100)/0.1 mM EGTA/1 mM dithiothreitol (pH 7) to remove

Abbreviations used: AS, anthroyloxystearic acid; PLC, mammalian phosphoinositide-specific phospholipase C; POPG, 1-palmitoyl-2-oleoyl-phosphoglycerol; PtdCho, 1-palmitoyl-2-oleoylphosphatidylcholine; PtdEtn, 1-palmitoyl-2-oleoylphosphatidylethanolamine; PtdSer, 1-palmitoyl-2-oleoylphosphatidylserine.

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buffer. 7-Hydroxycoumarin was purchased from Molecular Probes (Eugene, OR, U.S.A.). PtdIns(4,5) P_2 was prepared by extraction from Folch I fractions purchased from Sigma (St. Louis, MO, U.S.A.) [10]. Detergents were purchased from Boehringer and lipids were from Avanti Polar Lipids (Alabaster, AL, U.S.A.). PLC- δ_1 was labelled with acrylodan (Molecular Probes) by solubilizing the protein in buffer without reducing agents, adding a 4-fold molar excess of probe and incubating on ice for 30 min. The mixture was dialysed against three changes of a 500-fold volume excess of buffer [20 mM Hepes/150 mM KCl/1 mM dithiothreitol (pH 7.0)].

Stock solutions of mixed micelle substrates containing 2.5, 5 and 10 mol % PtdIns(4,5) P_2 or 1-palmitoyl-2-oleoyl-phosphoglycerol (POPG) were made by drying the lipids under nitrogen and redissolving them in 0.1 M dodecylmaltoside and Chelex-treated NaCl (150 mM). The mixed micelles were stored under nitrogen at 4 °C. Stock solutions of large, unilamellar vesicles containing 57 % 1-palmitoyl-2-oleoyl phosphatidylethanolamine (PtdEtn), 10 % 1-palmitoyl-2-oleoyl phosphatidylcholine (PtdCho) and either 33 % PtdIns(4,5) P_2 or 33 % POPG were made by mixing the lipids in chloroform/methanol (1:1), drying them under nitrogen and then in a rotary evaporator, rehydrating them with 150 mM Chelex-treated NaCl and freeze-thawing them several times before extrusion through a 0.1 μ m filter.

RESULTS

Development of a real-time assay to monitor PLC activity by fluorescence

To determine the effect of hydrostatic pressure on the enzymic activity, we developed a real-time assay that monitored the rate spectroscopically while the sample was subjected to pressure.

Scheme 1 Reaction scheme of PtdIns(4,5) P_2 [PI(4,5) P_2] catalysis by PLC- δ

The pK values for the 4 and 5 positions of PtdIns(4,5) P_2 are 6.6–7.0 and 7.5, and the pK values for the 1, 4 and 5 positions of Ins(1,4,5) P_3 are 6.3, 5.7 and 7.8 respectively [24–26]. Abbreviation: DAG, diacylglycerol.

Figure 1 Spectroscopic assay of PtdIns(4,5) P_2 hydrolysis catalysed by PLC- δ_1

Examples of the real-time PLC- δ_1 assays in which the rate is monitored by the decrease in emission intensity, corresponding to a decrease in pH, of the indicator 7-hydroxy-4-methylcoumarin as protons are generated from PtdIns(4,5) P_2 catalysis (lower trace in upper panel) relative to that from control lipid (upper trace in upper panel) in detergent mixed micelles, and at different PtdIns(4,5) P_2 concentrations (lower panel).

The PtdIns(4,5) P_2 hydrolysis reaction catalysed by PLC- δ_1 (Scheme 1) generates a phosphate product with a low pK , so at neutral pH at least one proton will be released for each PtdIns(4,5) P_2 hydrolysed. Thus, by conducting the PLC- δ_1 -catalysed reaction in weakly buffered solution, we could monitor proton release using the fluorescent pH indicator 7-hydroxy-4-methylcoumarin, which has a pK^* of approx. 7.6. Under the conditions used here, we observed a decrease in emission intensity to approx. 11 % on deprotonation of the probe.

Real-time activity studies were measured by placing the substrate in a working solution containing all components except enzyme (150 mM Chelex-treated NaCl/18 μ M $CaCl_2$ /0.9 mM dithiothreitol/0.1 μ M 7-hydroxy-4-methylcoumarin, with substrate or control lipid embedded in micelles or bilayers). A small amount of NaOH was added to bring the pH to 7.7–8.0, which is the most sensitive range of dye response. The enzymic activity of PLC- δ_1 is constant over this pH range [11] implying that

Table 1 K_m values for PLC- δ determined by fluorescence

The bilayers were PtdEtn/PtdIns(4,5) P_2 (2:1), and the assay conditions were as described in the text; $n = 2-4$. Estimated errors ranged from 0.1 to 0.4 and from 0.01 to 0.03 for V_{max} and K_m respectively.

System	[PLC- δ] (nM)	[PtdIns(4,5) P_2] (%)	V_{max} (nmol/s)	K_m (mM)
Micelles	200	10	-3.1	0.17
	200	5	-1.8	0.16
	200	2.5	-0.8	0.18
	100	10	-1.6	0.10
	200	10	-3.1	0.19
Bilayers	200	10	-1.0	0.05

enzyme-substrate interactions are not perturbed. The solution was placed in a cuvette and the fluorescence was monitored with excitation at 390 nm and emission at 475 nm. After a stable baseline had been recorded, the reaction was initiated by adding unbuffered enzyme to the cuvette while stirring under N_2 and measuring the change in emission intensity as a function of time using a non-substrate lipid, POPG, as a control (see Figure 1). At the end of the assay, a known equivalent of base was added to the reaction solution to calibrate the response. Initial studies were performed with lipid substrates dispersed in dodecylmaltoside micelles.

We measured the reaction rates at different substrate and enzyme concentrations (Table 1) and found the results mirrored those obtained by radiometric assays (results not shown). When substrate incorporated into PtdCho/PtdSer/PtdEtn bilayers were tested, the change in emission intensity was somewhat diminished, possibly owing to buffering by the lipid headgroups.

Michaelis-Menten parameters were calculated from the assay curves. Because the change in fluorescence intensity of the probe is assumed to be directly proportional to the amount of product formed and we know the initial amount of substrate, $[S]_0$, the concentration of substrate at any time during the reaction, $[S]_t$, is calculated by comparing the change in intensity with the change caused by the addition of a known amount of base at the end of the experiment. The Michaelis-Menten parameters are determined from the integrated rate equation:

$$V_{max}t = (K_m \ln[S]_0/[S]_t) + [S]_0 - [S]_t$$

Graphically, V_{max} (app) corresponds to the initial slope; $[S]_0$ and $[S]_t$ correspond to the initial and final intensities per mol of H^+ . In Table 1 we give the apparent V_{max} and K_m values obtained from these curves.

Effect of pressure on PLC- δ enzymic activity

We determined the effect of pressure on the activity of PLC- δ with PtdIns(4,5) P_2 incorporated into micelles and bilayers using a home-built pressure cell based on the design of Paladini and Weber [12]. These studies were performed by initiating the reaction at 5 °C to decrease the reaction rate, enabling us to assemble and pressurize the sample (total delay time approx. 2 min). We found the emission intensity of the probe to be sensitive to pressure, increasing by 2%/100 MPa, but this response occurred during the dead time of the pressurization and did not change over a 30 min period (the longest duration) at constant pressure. The result of a typical assay is shown in Figure 2. Rather than the decrease in activity to one-third or one-

Figure 2 Effect of pressure on the rate of PtdIns(4,5) P_2 hydrolysis by PLC- δ_1

Shown is the pressure response of the PtdIns(4,5) P_2 hydrolysis assay in lipid bilayers containing 33% PtdIns(4,5) P_2 at 10 °C; the arrows indicate the time at which pressurization began and the time at which maximum pressure was achieved. Abbreviations: FI, fluorescence intensity; a.u., arbitrary units.

quarter predicted from monolayer studies (see the Discussion section), we observed an increase in the reaction rate with pressure (Figure 3). All pressure curves showed the expected reversal in rate as the pressure was lowered. Clearly, the increase in lipid packing brought about by high pressure did not decrease the activity of the enzyme.

Two types of control study were done to improve our understanding of the increase in PLC- δ activity under pressure. First, we explored the possibility that activation resulted from enhanced membrane binding under pressure: we measured the binding of PLC- δ to PtdCho/PtdIns(4,5) P_2 (95:5) spectroscopically by doping the membranes with 1% dansyl-PtdEtn and adding the enzyme. Dansyl-PtdEtn is efficiently quenched by

Figure 3 Increase in the rate of PtdIns(4,5) P_2 hydrolysis with pressure

The percentage increase in rate, calculated by the difference between the rate at elevated pressure minus the rate at atmospheric pressure divided by the atmospheric pressure value, is shown as a function of pressure for PtdIns(4,5) P_2 in detergent mixed micelles (●) and bilayers (■) at 10 °C. The error bars represent S.D.

Figure 4 Membrane penetration of PLC- δ as measured by fluorescence energy transfer

Relative energy transfer from PLC- δ Trp to an AS series, in which the anthroyl probe was located on the 2-, 6-, 9-, 12- and 16- positions, as measured by the relative intensity of emission of the anthroyl moiety when excited through Trp donors ($\lambda_{\text{ex}} = 280$ nm) relative to direct excitation ($\lambda_{\text{ex}} = 381$ nm) with 300 μM PtdCho/PtdSer (2:1) labelled with 1% AS probes and 100 nM PLC- δ_1 . Results are shown relative to the intensity in the absence of protein, which was set to zero. FI, fluorescence intensity.

water [13] and its emission intensity increases by approx. 40% when PLC- δ binds to the membrane surface and displaces water. A comparison of the change in intensity of dansyl-PtdEtn membranes under pressure in the absence of PLC- δ , under conditions in which PLC- δ is partly bound, and under conditions in which the PLC- δ is fully bound allowed us to calculate the change in the percentage bound while pressurizing the cell from atmospheric pressure to 200 MPa. We found that pressure did not alter PLC- δ_1 binding (results not shown). Complementary studies that measured the binding of PLC- δ_1 labelled with a fluorescent probe (acrylodan) to unlabelled lipid bilayers gave identical results.

We also explored the possibility that the increase in enzyme activity under pressure was due to an increase in the hydration of the membrane surface, because we have previously observed that the incorporation of sphingomyelin lipids, which dehydrate the membrane surface, decreases PLC- δ_1 activity. In these studies, we doped PtdCho/PtdIns(4,5) P_2 (67:33) membranes with 1% dansyl-PtdEtn and compared the change in fluorescence intensity of the dansyl residue in the presence of water, which quenches its fluorescence, with that of $^2\text{H}_2\text{O}$, which does not. We found that the hydration of the dansyl probe did not change under pressure (results not shown).

Penetration of PLC- δ into the lipid bilayer by fluorescence energy transfer

Because monolayer studies indicate the penetration of PLC- δ into the membrane surface, we measured the extent of this penetration by fluorescence energy transfer. Here we measured the relative amount of energy transfer from Trp donors in PLC- δ to a series of anthroyloxystearic acid (AS) probes that were located at increasing depths in the membrane bilayer. The distance at which 50% energy transfer occurred was approx. 2 nm [14]. Energy transfer was assessed by comparing the area under the emission curve of AS when only the anthroyl moieties were excited with that when both the anthroyl and Trp moieties were excited. The results are shown in Figure 4. Most of the energy transfer occurred from the PLC- δ Trp to 2-AS and 6-AS,

which are located close the membrane surface. For probes that were more deeply buried, the extent of transfer was smaller. From the crystal structures of the N-terminus [15] and catalytic domains [16], the only Trp residues that are most likely to transfer energy to the more deeply buried probes were those in the hydrophobic rim of the catalytic domain; if the proteins penetrated significantly, then we would expect a greater extent of transfer to these buried probes. The data in Figure 4 indicate a shallow membrane penetration.

DISCUSSION

PLC- δ is an interfacial enzyme that must access its lipid substrate during catalysis; previous monolayer studies suggest that access occurs by penetration into the membrane. In the present study we used high pressure to determine the importance of lipid packing in enzyme catalysis. Because the crystal structures of the N-terminus of PLC- δ and the remaining C-terminal portion of the enzyme have been solved, we can postulate the regions of the protein that might be responsible for membrane penetration. The N-terminal pleckstrin homology (PH) domain has been shown to have a key role in PLC- δ membrane binding through specific interactions with PtdIns(4,5) P_2 [17,18]. Its crystallographic structure indicates that specific binding to PtdIns(4,5) P_2 need not involve significant penetration of this region of the protein [19]. In addition, the crystal structure of the catalytic domain indicates that the EF hands do not interact with the membrane surface. The C2 domain of the enzyme, which is directly linked to the catalytic core, does not seem to be involved in membrane binding [19]. Thus the region in which membrane penetration is likely to occur is the catalytic region. The active site of PLC- δ is a broad depression with a maximal depth of 1 nm containing a hydrophobic rim that is suspected to insert into the membrane [16]. Supporting this idea is the result that replacing Phe-360 with Ala enhances PLC activity in monolayers, implying that the mutated enzyme could penetrate the interface more easily [20]. Although the involvement of a hydrophobic ridge surrounding the active site seems attractive, it is unclear whether any portion of the protein actually penetrates the non-polar core of the membrane or even dips down between the polar headgroups. Moreover, many of the hydrophobic residues identified on the rim of PLC- δ_1 are replaced by polar amino acids in the PLC- β and PLC- γ isoenzymes. Thus residues in this region might have a role in membrane recognition but not necessarily in penetration. This idea is consistent with energy-transfer results showing that Trp residues do not insert deeply into the membrane surface.

Penetration of the enzyme into the membrane should be inhibited by the application of hydrostatic pressure, yet we find that the enzymic activity increases significantly, in direct contrast with lateral-pressure studies. To understand this difference we first need to determine whether the studies sample the same range of membrane compression. We have done this by equating the compressibility of the membrane surface that occurs during the lateral-pressure studies to the compressibility of the membrane surface that occurs during the hydrostatic pressure measurements. The lateral compressibility can be expressed as:

$$\beta_{\text{lat}} = -1/A(dA/dp)$$

Integration and taking the natural logarithm gives:

$$\ln A/A_0 = -\beta_{\text{lat}}(p-p_0)$$

where A_0 and p_0 are the initial values of the area and pressure. From [5], at 20 mN/m the area of a PtdCho headgroup is 0.8 nm² and at 35 mN/m this area is decreased to 0.65 nm², yielding $\beta_{\text{lat}} = -0.0138$ (mN/m)⁻¹ at room temperature. The lateral com-

compressibility under hydrostatic pressure for PtdCho lipid vesicles has been reported as $-0.765/\text{GPa}$ at 22°C . Assuming that these compressibilities are linear with pressure in the range of interest, we can calculate the hydrostatic pressure needed to produce a decrease in area from 0.8 to 0.65 nm^2 from lateral pressure measurements [21]. This value, 260 MPa , is the hydrostatic pressure equivalent to the increase in lateral pressure from 20 to 35 mN/m and indicates that the monolayer studies went to a higher equivalent pressure than the hydrostatic pressure studies. Conversely, the 100 MPa range that we have used here is equivalent to an increase in lateral surface pressure of 6 mN/m .

Although the lateral-pressure studies accessed a decrease in surface area that was not explored here, our study should have detected a decrease to one-third if the forces acting on the protein were equivalent in the two techniques. Specifically, increasing the lateral pressure from 20 to 26 mN/m decreases the activity from 56 to $14\text{ mol of PtdIns(4,5)P}_2/\text{min per } \mu\text{g of PLC-}\delta$ [5]. If the mechanism causing this decrease in activity is similar to hydrostatic pressure, then the equivalent 0.1 – 1000 MPa change used here should give a similar decrease in activity. The change in enzymic activity with pressure is related to the activation volume (ΔV^*) by:

$$d[\ln(k_f/k_b)]/dp = -\Delta V^*/RT$$

where k_f and k_b represent the forward and backward reaction respectively.

We obtain a predicted value of $\Delta V^* = +30\text{ ml/mol}$ from the lateral-pressure studies. Instead, the activation volume calculated from our hydrostatic pressure studies (Figure 4) gives a ΔV^* of -14 ml/mol . Thus the rate-limiting steps must be different in the two techniques. In the monolayer studies, the membrane is being compressed while the protein is still at low pressures. Inactivation under these conditions is interpreted as being due to the inability of the enzyme to access substrate under more restricted conditions, which is the rate-limiting step. In the hydrostatic pressure studies both the protein and the membrane are under identical pressures and there is no net force that the enzyme must work against to access substrate, which might be a more appropriate model system. Our studies show that the increase in lipid packing does not decrease enzymic activity; access to substrate is not rate-limiting in bilayers. Supporting this idea is the observation that activation is similar whether substrate is embedded in membrane bilayers or detergent mixed micelles even though these aggregates have different compressibilities. This result means that the effect of hydrostatic pressure must result from the nature of the enzymic reaction rather than the nature of the environment surrounding the substrate. Thus monolayer studies represent the effect of an upwards lateral force of the protein as opposed to the effect of lipid packing.

Our control studies have shown that the increase in enzyme activity is not due to changes in surface hydration; previous work indicates that membrane surface charge does not change under pressure. Thus the idea that activation is not due to changes in membrane properties correlates well with the observation that PLC- δ membrane binding is unaffected by pressure or the volume change is zero, i.e.:

$$\Delta V = 0 = d(-RT\ln K_{eq})/dp = d(-RT\ln k_{off}/k_{on})/dp$$

Because the ratio of the off and on rates (k_{off}/k_{on}) is unchanged by pressure and the on rate is not expected to change because the viscosity of water is constant over this pressure range [22], then the off rate should also be unaffected by pressure. Keeping in mind that the enzyme will diffuse on and off the membrane

several thousand times per second, if the enzyme penetrated the membrane surface we should expect changes in the on or off rate; however, this does not seem to be so.

The effect of high pressure has been studied in a number of enzyme systems and in some cases, but not many, pressure causes deactivation but increases the rate of one of the reaction steps [8]. What is the cause of the pressure-induced activation of PLC- δ ? The application of pressure to a system will shift the equilibrium to the side that occupies the least volume. If the number of products is greater than reactants, pressure would in general cause a shift towards reactants, but because the number of bonds broken and formed are the same we do not expect pressure to affect the equilibrium based on the number of species. Although pressure might affect the amount of bound Ca^{2+} , under our assay conditions Ca^{2+} was in excess and if pressure caused an increase in Ca^{2+} binding it would not have been detected. A decrease in Ca^{2+} binding would result in a detriment in activity, which was not seen here. An activating factor could be electrostriction or the condensation of water around newly formed charges, which occurs with a volume change of -15 ml/mol [7]. Because the hydrolysis of PLC at neutral pH generates a tervalent anion and a proton, a similar volume change would be expected. Therefore we argue that pressure increases the reaction rate by increasing the rate of formation of ionized products. Whether this change is mediated directly by residues that contact product or others is uncertain because it has been shown that mutations in the N-terminal binding domain can affect the catalytic domain [23].

In summary, we have measured the activity of PLC- δ under hydrostatic pressure with the use of a novel real-time assay that might be applicable to other PLC studies. We find that lipid packing in bilayer membranes does not strongly influence enzyme activity under the conditions that we used, probably because the penetration of the enzyme into the membrane surface is shallow. Therefore, in fluid biological membranes that are not typically under stress, the packing of the lipid headgroups would not be expected to restrict access to substrate or dampen enzymic activity.

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