Binding of phospholipase C δ_1 to phospholipid vesicles

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Binding of phospholipase C δ_1 (PLC δ) to phospholipid vesicles was studied using large, unilamellar phospholipid vesicles (LUVs). PLC δ bound weakly to vesicles composed of phosphatidylserine (PS) or phosphatidylcholine (PC) or phosphatidylethanolamine (PE)+PC, and even more weakly to vesicles composed of phosphatidylinositol. The enzyme bound strongly to LUVs composed of PE+PC and phosphatidylinositol 4,5-bisphosphate (PIP $_2$) or sphingomyelin (SM). Binding of 50% of PLC δ occurred at 0.25 nmol/ml PIP $_2$ when LUVs composed of PE+PC (molar ratio of 80:20), plus various amounts of PIP $_2$, were used at a constant phospholipid concentration of 300 nmol/ml. When LUVs composed of PE+PC+PIP $_2$ (molar ratio of 79:20:1) were tested as a function of increasing phospholipid concentration, 50% binding of PLC δ occurred at 1.2 nmol/ml PIP $_2$ and 120 nmol/ml total phospholipid. Similar measurements were conducted with other phospholipids and PIP $_2$ at a molar ratio of 99:1. These showed that 50% binding

of PLC δ occurred at a level of 0.9 nmol/ml PIP $_2$ with 80 nmol/ml PC; at 2.2 nmol/ml PIP $_2$ with 170 nmol/ml PS; at 4.2 nmol/ml PIP $_2$ with 320 nmol/ml PI; and at 0.26 nmol/ml PIP $_2$ with 20 nmol/ml total liver phospholipids. Binding to phosphatidylinositol 4-phosphate was much weaker. When LUVs composed of PE+PC+SM (molar ratio 48:12:40) were tested as a function of increasing phospholipid concentration, 50% binding of PLC δ occurred at a level of 96 nmol/ml SM. This is well below the concentration of SM that can be calculated to face the cytosol. Binding of PLC δ to LUVs decreased as the temperature was lowered from 37 °C to 0 °C. Thus PLC δ shows a high degree of specificity for binding to PIP $_2$ and SM. Under physiological conditions a considerable fraction of PLC δ may be bound to cellular membranes, either in an inactive form if bound to PIP $_2$ at low resting Ca $^{2+}$ concentrations, or in the inhibited form if bound to SM.

INTRODUCTION

Four major types of phosphoinositide-specific phospholipase C (PLC) called α , β , γ , and δ have been characterized [1–4]. These different forms of PLC all hydrolyse phosphatidylinositol 4,5-bisphosphate (PIP $_2$). They are also capable of hydrolysing phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol (PI), but the extent to which they do so under physiological conditions is uncertain. The purified isoforms of PLC are soluble, but in cell homogenates substantial amounts are found in membrane-bound forms [5–8]. For example, immunoassays indicate that a major fraction of PLC α and PLC γ is associated with the cell membrane [1,8]. The different isoforms of PLC are regulated differently. Incubation of certain types of cells with epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) results in the phosphorylation of PLC γ , but not of PLC β and PLC δ [9–12]. Treatment of A-431 cells with EGF causes a redistribution of PLC γ from the cytosol to the cell membrane [13]. Thus phosphorylation appears to affect the intracellular distribution of PLC γ . When PLC γ is immunoprecipitated from extracts of EGF- or PDGF-treated cells, a similar spectrum of co-precipitated proteins is observed, suggesting that the association of PLC γ with membranes may be mediated by other proteins [9,10,12]. Since many cells possess more than one isoform of PLC [3,9,14], it seems likely that there exist different mechanisms for regulating the interaction of these enzymes with cell membranes. For example, it was shown that profilin may compete with cytosolic PLC γ for interaction with membrane-bound PIP $_2$ [15]. If this is the case, similar competition may occur with other isoforms of PLC.

We show here that PLC δ binds to phospholipid vesicles in a manner that depends critically on the phospholipid composition of the vesicles and on the temperature. PLC δ binds strongly to phospholipid vesicles containing PIP $_2$ or sphingomyelin (SM). Much weaker binding is also observed when the vesicles contain PIP, although PIP $_2$ and PIP are equally good substrates in the detergent-micelle assay [16]. PLC δ binds weakly to vesicles composed of other phospholipids.

MATERIALS AND METHODS

Materials

Crude phosphoinositide mixture and cholesterol were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [2- 3 H]PIP $_2$ (4.8 Ci/mmol) was purchased from Du Pont–New England Nuclear (Boston, MA, U.S.A.). Unlabelled PI, phosphatidylserine (PS), phosphatidylethanolamine (PE), cardiolipin, and SM were from Avanti Polar Lipids, Inc. (Birmingham, AL, U.S.A.). PLC δ was purified from rat liver as described previously [16]. Analyses of tryptic peptides showed this to be the δ_1 isoform of PLC (M. Haber and J. M. Lowenstein, unpublished work).

Methods

Unlabelled PIP and PIP $_2$ were purified from a crude phosphoinositide mixture by t.l.c., according to the method of Jolles et al. [17]. This method was also used for analytical separations of PIP $_2$, PIP, and PI. Total rat liver lipids (TLL) were extracted by the method of Folch et al. [18] and stored in chloroform/methanol under N $_2$ or Ar. Large unilamellar phospholipid vesicles (LUVs)

Abbreviations used: LUVs, large unilamellar phospholipid vesicles; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP $_2$, phosphatidylinositol 4,5-bisphosphate; PLC δ , phospholipase C, isoform δ_1 ; PS, phosphatidylserine; SM, sphingomyelin; TLL, total rat liver lipids; EGF, epidermal growth factor; PDGF, platelet-derived growth factor.

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were prepared in 0.3 M sucrose using the method of Mueller and Chien [19]. After the swelling period, the vesicles were harvested by adding 2 vol. of 50 mM K/Hepes buffer, pH 7.4, and centrifuging at 12000 *g* for 10 min. The vesicles formed a loose pellet which was suspended in the original volume of buffer and centrifuged again. This washing procedure was repeated twice, after which the vesicles were suspended in the desired medium. The vesicles were prepared at 6 °C, except in the case of vesicles containing SM, which were prepared at 50 °C. Vesicles were stored at 6 °C and used for binding studies for up to two days after preparation, except for vesicles containing SM, which were used on the same day.

Phospholipid concentrations were determined after perchloric acid digestion [20] by measuring orthophosphate [21]. Phospholipid vesicle concentrations are expressed as μmol of lipid phosphate/ml. Protein was determined by the method of Bradford [22] using BSA as standard, or by measuring the absorbance at 280 nm.

Binding experiments were performed in final concentrations of 50 mM Na/Hepes buffer, pH 7.4, 100 mM KCl, 1 mM dithiothreitol, and 300 μM EGTA, hereafter called buffer A. Various amounts of phospholipid vesicles and enzyme, typically 120 ng/ml, were added to buffer A to yield a final volume of 0.15 ml. After incubation for 5 min at the temperature noted in the figure legends, 0.1 ml of the mixture was centrifuged at the same temperature at 40000 *g* in a DuPont-Sorvall RC2-B centrifuge for 7 min. The pellet was resuspended in 0.1 ml of buffer A and the activity of PLC δ was measured as described below. Results are expressed as $100 \times (\text{enzyme bound}/\text{total enzyme activity in the incubation medium without centrifugation})$. Under these conditions, the phospholipid concentrations before and after centrifugation and resuspension were the same. Controls, in which the centrifuged LUVs were resuspended in the supernatant instead of fresh buffer A, gave 95–98 % recovery of activity. The recovery of phospholipid vesicles during centrifugation was 96–98 % as determined with vesicles containing [2-³H]inositol-labelled PIP₂. All incubations and centrifugations were performed in plastic tubes to prevent loss of enzyme activity, which was found to occur in glass tubes.

PLC δ activity was assayed as described previously [16] using 17 μM [2-³H]inositol-labelled PIP₂ (14000 d.p.m./nmol), 2.4 mM deoxycholate, 300 μM CaCl₂, 100 μM EGTA, 90 mM KCl, 100 mM NaCl, 200 μM spermine, and 50 mM K/Hepes buffer, pH 7.4. The reaction was started by adding enzyme and was run at 37 °C for 3 min.

RESULTS

PLC δ binds weakly to LUVs prepared from pure phosphatidylcholine (PC), or PS, or a mixture of PE + PC (80:20, mol %), and even more weakly to vesicles prepared from pure PI (Figure 1). Binding of PLC δ to the vesicles is much stronger when they contain 1 mol% of PIP₂ (Figure 2). For example, incubation of PLC δ with vesicles containing PE + PC + PIP₂ (79.8:19.2:1, mol %) results in 82 % binding in the presence of 0.6–0.8 $\mu\text{mol}/\text{ml}$ total phospholipid. Similar behaviour is observed with vesicles containing PIP₂ and either PC, or PS, or PI; however, the maximum amount of PLC δ bound is then only 35 %, suggesting that PE plays a supportive role in the binding process (Figure 2). Hydrolysis of PIP₂ by PLC δ is stimulated 50–60 % by PE in both the detergent and liposome assays in the absence of spermine [23]. At the PLC δ concentration used (120 ng/ml), the phospholipid concentration required for achieving 50 % of maximum binding to PE + PC vesicles containing 1 % PIP₂ is 0.12 $\mu\text{mol}/\text{ml}$. Vesicles composed of the other phospholipids bind PLC δ less

strongly (Figure 2). In an attempt to set the binding of PLC δ to LUVs in a more physiological context, we tested the effect of TLL. Binding of PLC δ to vesicles prepared from TLL is also weak (Figure 3). When vesicles are prepared so that they contain TLL + PIP₂ (98.7:1.3, mol %), binding of PLC δ is 100 % at a level of 0.20 $\mu\text{mol}/\text{ml}$ total phospholipid (Figure 3). When PIP is substituted for PIP₂, the binding is weaker and only 54 % of PLC δ is bound to the vesicles at the relatively high concentration of 1 $\mu\text{mol}/\text{ml}$ phospholipid. The concentrations of TLL + PIP₂ and TLL + PIP required for 50 % binding of enzyme are 0.02 and 0.17 $\mu\text{mol}/\text{ml}$ respectively. Very little enzyme is bound to the vesicles prepared from TLL + PI (Figure 3). Our TLL preparation did not contain detectable amounts of PIP₂, as checked by t.l.c., presumably because most or all of the PIP₂ was degraded during

Figure 1 Binding of PLC δ to phospholipid vesicles lacking PIP₂

The phospholipid vesicles consisted of $\circ$, PC; ∇ , PS; $\square$, PI; and $\triangle$, 80% PE + 20% PC. Incubations were conducted at 22 °C.

Figure 2 Binding of PLC δ to phospholipid vesicles containing 1 mol% PIP₂

Conditions were as for Figure 1, except that the phospholipid vesicles also contained 1 mol% PIP₂.

Figure 3 Binding of PLC δ to phospholipid vesicles prepared from TLL

The vesicles consisted of: ●, TLL + 1.3 mol% PIP $_2$; ▼, TLL + 1.3 mol% PIP; ■, TLL + 1.3 mol% PI; ▲, TLL without added phosphoinositides. Incubations were conducted at 22 °C.

Figure 4 Binding of PLC δ to phospholipid vesicles as a function of the PIP $_2$ and PIP concentrations

The phospholipid vesicles consisted of 80% PE + 20% PC at a concentration of 300 nmol/ml. The vesicles also contained PIP $_2$ or PIP at the indicated concentrations. Note that concentrations are in nmol/ml.

the isolation of the phospholipid fraction from whole liver. A significant amount of PIP $_2$ is degraded < 1 s after removal of the tissue [24,25].

When vesicles composed of PE + PC (80:20, mol%) are used at a constant phospholipid concentration of 300 nmol/ml, and the PIP $_2$ concentration is increased, 50% binding of PLC δ occurs at a level of 0.21 nmol/ml PIP $_2$ (Figure 4). When PIP $_2$ is replaced by PIP, binding of PLC δ is much weaker. Binding of enzyme to the TLL + PIP $_2$ vesicles is weaker at 0 °C and 22 °C

Figure 5 Temperature dependence of the binding of PLC δ to phospholipid vesicles

The vesicles consisted of TLL + 1.3 mol% PIP $_2$. Enzyme was incubated with phospholipid vesicles at: ■, 0 °C; ▲, 22 °C; and ●, 37 °C.

Figure 6 Binding of PLC δ to phospholipid vesicles in the presence of SM

The vesicles consisted of: △, 80% PE + 20% PC; ■, 20% SM + 64% PE + 16% PC; and ●, 40% SM + 48% PE + 12% PC. Incubations were conducted at 22 °C.

than at 37 °C (Figure 5). The concentrations of TLL + PIP $_2$ required for 50% of maximum binding of PLC δ are 0.007, 0.020 and 0.025 μ mol/ml at 37 °C, 22 °C and 0 °C respectively. These results demonstrate a high affinity of PLC δ for LUVs containing PIP $_2$.

SM is a strong inhibitor of PLC δ [23]. For this reason we tested binding of the enzyme to LUVs containing SM. With vesicles composed of SM + PE + PC (40:48:12, mol%), 50% of maximum binding is observed at 112 nmol/ml SM and 280 nmol/ml total phospholipid (Figure 6).

The inside-versus-outside distribution of PIP₂ and PIP in the phospholipid bilayer of LUVs was determined as follows. LUVs composed of PE + PC + phosphoinositide (79.8:19.2:1.0, mol %) were incubated with PLC δ at 37 °C until no further inositol triphosphate or inositol bisphosphate was liberated. This was determined by running assays as a function of time. With the amount of PLC δ employed, this typically took 5 min. Deoxycholate was then added to a final concentration of 2.4 mM and the incubation was continued until all of the phosphoinositide had been cleaved. This typically took 5 min. The results showed that 85 % of PIP₂ and 71 % of PIP faced outward from the LUVs.

DISCUSSION

PLC δ exhibits a high degree of binding specificity for PIP₂ and SM. Binding of PLC δ to lipid vesicles not containing PIP₂ or SM is weak and depends on the composition of the phospholipids that make up the vesicles. Animal cells typically contain about 3 mg of phosphoinositides/g fresh weights of which about 1 % is PIP₂ [26]. This is equivalent to 27 nmol of PIP₂/g fresh weight. It is assumed that in intact cells all of the PIP₂ faces the cytosol, an assumption which is reasonable because the kinases that phosphorylate PI are intracellular enzymes. Assuming that cytosol accounts for 50 % of the fresh weight, this yields 54 nmol of PIP₂/ml of cytosol. This is much higher than the concentration of PIP₂ in LUVs required for 50 % binding of PLC δ , namely 0.25 nmol of PIP₂/ml (Figure 3). Corrected for the fraction of PIP₂ that faces outward from the LUVs, namely 85 %, this becomes 0.21 nmol of PIP₂/ml. On the basis of these considerations, most or nearly all of the PLC δ would be expected to be bound to membranes via PIP₂. PLC δ would have to be bound in an inactive form, otherwise it would hydrolyse its substrate rapidly. Alternatively, a different protein such as profilin may block binding of PLC δ to PIP₂, or bound enzyme may be partitioned between PIP₂ and SM (see below).

Results on the distribution of SM in hepatocytes are somewhat uncertain and the following should be considered with this in mind. In the hepatocyte, SM occurs in lysosomal, plasma and Golgi membranes, while significant amounts of SM are also found in rough endoplasmic reticulum, and in nuclear and mitochondrial membranes [27]. However, such results probably reflect contamination of cell fractions by dispersed plasma membrane fragments [28]. The total amount of SM, as well as its intracellular distribution, changes in various diseases (reviewed in [29]). SM is asymmetrically distributed in the lipid bilayer of plasma membranes. The asymmetry differs widely depending on the cell type [30–32]. The total phospholipid content of rat liver is 25 mg/g wet weight, of which 8.2 % is SM [33]. This is equivalent to 2.8 μ mol of SM/g fresh weight. Assuming that 20 % of the SM faces inward, then $2.8 \times 0.20 = 0.56 \mu$ mol of SM/g of liver may face the cytosol. This is equivalent to 1.1 μ mol of SM/ml of cytosol, if it is assumed that cytosol accounts for 50 % of the fresh weight. This is about 10 times greater than the concentration of SM required for 50 % binding of PLC δ in the experiments shown in Figure 6. In erythrocytes and Friend erythroleukemia cells, 15–20 % of the plasma membrane SM faces the cytosol [31,32]. Calculations similar to the above show that this is equivalent to 0.4–0.6 μ mol of SM/ml of cytosol. This is 4–6 times greater than the concentration of SM required for 50 % binding of PLC δ . On the basis of this limited information, we conclude that inward-facing SM may be a factor involved in the binding of PLC δ to the membranes of liver and other tissues.

The degree to which PIP₂ and SM compete for binding to PLC δ remains to be established.

Turnover and net decline of SM have been observed in response to various extracellular signals, including vitamin D₃, and tumour necrosis factors α and γ . The maximum decline of SM observed in HL-60 cells occurred in 1–2 h and was about 40 % of the total SM. The original levels of SM were restored after 1–4 h [34]. Although the organelles in which the decline in SM occurs and the sidedness of the decline were not investigated, these observations point to a possible diminution in the inhibition of PLC δ by SM under certain physiological conditions.

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