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Regulation of phospholipase C δ1 by sphingosine

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Abstract

Sphingosine, which is on the pathway of sphingomyelin degradation, activates phospholipase C (PLC) δ1 moderately. In the liposome assay effect of sphingosine on PLC δ1 activity depends on KCl concentration. Stimulation of PLC δ1 by sphingosine increased as the KCl concentration is increased from 0 to 100 mM, and then diminished with the increasing KCl. In the liposome assay sphingosine diminishes inhibition of PLC δ1 by sphingomyelin. To determine the domain of PLC δ1 which interacts with sphingosine active proteolytic fragments of PLC δ1 were generated by trypsin digestion of the native enzyme. Sphingosine affects the activity of PLC δ1 fragment which lacked the amino-terminal domain (first 60 amino acids) but not the active fragment that has cleaved the domain spanning the X and Y region of PLC δ1. These observations indicate that for interaction of sphingosine with PLC δ1 intact domain that span regions of conservation, designated as X and Y is necessary. When the activity of PLC δ1 was assayed with PIP_2 in the erythrocyte membrane as substrate, sphingosine strongly inhibited PLC δ1. The other homolog of sphingosine 4-hydroxysphinganine (phytosphingosine) inhibited PLC δ1 to much lesser extent. The activity of PLC δ1 was inhibited by 68% and 22% in the presence of 20 μM sphingosine and phytosphingosine, respectively. This inhibition was completely abolished by deoxycholate at a concentration of 1.5 mM. These observations suggest that sphingosine may regulate activity of PLC δ1 in the cell.

Keywords: Phospholipase C δ1; Sphingosine; Erythrocyte membrane

1. Introduction

Three major types of phosphoinositide-specific phospholipase C (PLC), called β, γ and δ, have been characterized [1,2]. 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 [3–6]. The different isoforms of PLC are regulated differently. PLC γ appears to be regulated by tyrosine phosphorylation in response to EGF, FGF, NGF and PDGF receptor occupancy [7]. The phosphorylation of PLC γ does not affect the

Abbreviations: PLC, phospholipase C; SM, sphingomyelin; Sph, sphingosine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PIP_2, phosphatidylinositol-4,5-bisphosphate; PIP, phosphatidylinositol-4-phosphate; TLL, total rat liver lipids; IP_3, inositol-1,4,5-triphosphate; FGF, fibroblast growth factor; NGF, nerve growth factor; PDGF, platelet-derived growth factor; RhoGAP, Rho-specific GTPase activating protein

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kinetic properties of the enzyme, but causes a redistribution of the enzyme from cytosol to cell membrane [8,9]. Tyrosine phosphorylation of PLC γ promotes its association with actin components of the cytoskeleton [10,11]. PLC β isozymes are activated by the α and $\beta\gamma$ subunits of the heterotrimeric G proteins [12–14]. The receptors that are known to activate PLC β by α subunit of Gq are those for bradykinin, angiotensin II, thromboxane A2, vasopressin and acetylcholine [12]. The receptors for interleukin-8 and m2 and m4 subtypes of muscarinic acetylcholine receptor activate PLC β by $\beta\gamma$ subunits of G proteins [12,15–17]. The activation mechanism of PLC δ isozymes is not known at the present time. Banno and colleagues [18] reported that thrombin-stimulated phophoinositide hydrolysis in Chinese hamster ovary cells overexpressed PLC δ1 was dependent on proteins G and calcium. Recently, Homma and Emori reported that PLC δ1 binds to a novel RhoGAP protein [19]. This may suggest that in regulation of PLC δ1 may be involved the Rho signalling pathway. Previously it has been reported that sphingomyelin is a strong inhibitor of PLC δ1 and that this enzyme binds strongly to phospholipids vesicles containing PIP_2 or sphingomyelin [20–22]. Turnover and net decline of SM have been observed in response to extracellular signals, including 1 α, 25-dihydroxyvitamin D_3 [23], tumor necrosis factor α [24], γ-interferon [25], interleukin-1 [26,27], arachidonate [28], and brefeldin A [29]. Recently Linardic and Hannun reported that exposing U937 leukemia cells to TNF-α results exclusively in hydrolysis of sphingomyelin located within the inner leaflet of the plasma membrane [30]. Hydrolysis of sphingomyelin results in generation of ceramide which may be converted to sphingosine by ceramidase [31]. Sphingosine is a potent inhibitor of protein kinase C in vitro and of cellular events dependent on this enzyme [32]. Many systems have been found to be affected by sphingosine including inhibition of oxidative burst in human neutrophils, the release of arachidonic acid, PAF formation, expression of viral genes and cell growth and differentiation in several cell types [32,33]. Previously it has been reported that sphingosine moderately activates PLC δ1 in the absence of spermine [20]. Here we showed that sphingosine stimulates PLC δ1 in detergent and liposome assay. For sphingosine interaction with PLC δ1, intact domain that span the regions of conservation named X and Y is necessary. However, in assay with PIP_2 located in erythrocyte membranes, sphingosine is a strong inhibitor of PLC δ1 activity. We postulate that in interaction of PLC δ1 with plasma membranes besides PIP_2 other phospholipids are involved and sphingosine competes with them for the same binding site within the PLC δ1 molecule.

2. Materials and methods

Spermine, leupeptin, soybean trypsin inhibitor, penicillin, adenine, sphingosine, phytosphingosine, and crude phosphoinositide mixture were from Sigma-Aldrich Sp. z o.o. (Poznan, Poland). Pefabloc SC, trypsin (sequencing grade) were from Boehringer Mannhein GmbH Biochemica (Mannhein, Germany). [2-^{3}H]Inositol-PI-4,5-P_2 (4.8 Ci/mmol) and [^{32}P]orthophosphoric acid were purchased from Du Pont GmbH (Dreieich, Germany). Unlabeled phosphatidylinositol, phosphatidylethanolamine, phosphatidylcholine and sphingomyelin were from Avanti Polar Lipids, Inc. (Birmingham, AL, USA).

Unlabeled PIP and PIP_2 were purified from a crude phosphoinositide mixture by thin-layer chromatography according to the method of Jolles et. al. [34]. This method was also used for analytical separation of PIP_2 and PIP. Phospholipid concentrations were determined after perchloric acid digestion [35] by measuring orthophosphate [36]. Protein was determined by the method of Bradford [37] using bovine serum albumin as standard, or by measuring the absorbance at 280 nm.

PLC δ1 was prepared from rat liver as described previously [38]. Purified enzyme reacts with rabbit anti-PLC δ1 but not with anti-PLC δ3 nor anti-PLC δ2 polyclonal antibodies.

2.1. Proteolysis with trypsin

Purified rat liver PLC δ1 (30–50 μg) was incubated with trypsin (at a molar ratio of PLC δ1:trypsin of 25:1) for different periods of time in 50 mM Hepes-NaOH buffer (pH 7.2), 5 mM $CaCl_2$, 5 mM DTT, 100 mM NaCl, 2.4 mM sodium deoxycholate at 24°C. The reaction was stopped by the addition of soybean trypsin inhibitor. The active fragments of

PLC δ1 were purified on a Mono S FPLC column as described [38].

2.2. Sequencing

Purified active fragments of PLC δ1 have been subjected to SDS/PAGE (10%) according to Laemmli [39] and electroblotted onto Immobilion-P (Millipore). After blotting, the membrane was stained with Coomassie Brilliant Blue, destained with 50% methanol, 15% acetic acid, and washed with water. The excised bands were subjected to sequence analysis on Applied Biosystem (ABI) Pulsed™ Liquid Protein sequencer.

2.3. Preparation of labeled erythrocyte inside-out vesicles

Human erythrocytes were prepared as described previously from blood withdrawn from healthy volunteers [40]. Cells for labeling were resuspended in 150 mM NaCl, 5 mM sodium phosphate (pH 8.0), 0.5 mM EGTA, 10 mM glucose, 1 mM adenine, 5 mM inosine, 100 μg streptomycin/ml, 100 units penicillin/ml and incubated in the presence of $^{32}PO_4$ (20 μCi/ml cells) for 20 h [41]. Incubation was terminated by addition of ice-cold sodium phosphate (pH 8.0), 150 mM NaCl, 0.5 mM EGTA, sedimented and washed twice in the same buffer. White ghosts were prepared from ^{32}P-pre-labeled cells as described [42]. The 'inside-out' vesicles were prepared by incubation of white ghosts in 30 vols. 0.1 mM EGTA (pH 8.5) at 37°C for 30 min. Resultant vesicles were depleted of spectrin and actin. Removal of remaining peripheral proteins was achieved by incubation of inside-out vesicles at alkaline pH as described [40,43]. The alkaline-stripped vesicles were subsequently used for experiments and in the text are referred to as erythrocyte vesicles.

To measure changes in phospholipid content resulting from the different treatments described above, control and prepared ghost, i.e., inside-out vesicles or stripped vesicles were submitted to an acidic lipid extraction as described [41]. The phospholipid separation was performed by thin-layer chromatography according to the method of Jolles et al. [34]. Phospholipids were located on chromatography plate by staining in iodine vapor. Spots corresponding to identified phospholipids were scrapped and radioactivity and phospholipid concentration was measured.

2.4. Assays for PLC δ1

PLC δ1 activity was assayed as described in details previously [20]. The reaction mixture for detergent assay contained 17 nmol/ml [2-^{3}H]inositol-labeled phosphatidylinositol-4,5-bisphosphate (PIP_2) (0.005 μCi/nmol), 2.4 mM sodium deoxycholate, 180 mM NaCl, 100 μM $CaCl_2$, 100 μM EGTA, 50 mM HEPES-NaOH buffer (pH 7.2). The reaction was started by adding PLC δ1 and was run in a final volume of 0.1 ml at 37°C for 1 min. In this reaction mixture, the concentration of free Ca^{2+} was 2.2 μM as determined by the arsenazo method [44].

The reaction mixture for the liposome assay contained 25 nmol/ml [2-^{3}H]inositol-labeled phosphatidylinositol-4,5-bisphosphate (PIP_2) (0.01 μCi/nmol), 200 nmol/ml of 9:1 mixture of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) and other phospholipids as indicated in the figure legends, 180 mM NaCl, 100 μM $CaCl_2$, 100 μM EGTA, and 50 mM Hepes-NaOH buffer (pH 7.2). The concentration of free calcium was 2.2 μM. The reaction was started by adding enzyme and was run in a final volume of 0.1 ml at 37°C for 1 min.

The reaction mixture for the erythrocyte vesicles assay contained 80 nmol/ml erythrocyte vesicle phospholipids labeled with $^{32}PO_4$ (0.002 μCi/nmol), 120 mM KCl, 60 mM NaCl, 100 μM $CaCl_2$, 100 μM EGTA, 20 μM leupeptin, 0.2 mM Pefabloc SC and 50 mM HEPES-NaOH buffer (pH 7.2). The concentration of free calcium was 2.2 μM. The reaction was started by adding enzyme and was run in a final volume of 0.1 ml at 37°C for 5 min.

In all assays, the reaction was stopped by adding 0.1 ml of 1.2 N HCl, the mixture was vortexed, 0.5 ml chloroform-methanol (2:1) was added, and the mixture was vortexed again. The aqueous layer was separated, and an aliquot was taken for counting. Assay conditions were chosen so that the reaction rate was proportional to time and enzyme concentration. In all assays neither the substrate nor the inhibitor was in true solution. For this reason we prefer to quote their concentrations as nmol/ml. If substrate and inhibitor were in true solution, nmol/ml would become μM.

3. Results

Investigating the phospholipid dependence of PLC δ1, we used detergent and liposome assay. In the detergent assay substrate and examined phospholipid are present in the mixed deoxycholate micelles. The advantage of this assay is that it allows measurement of the activity of the enzyme that lacks the ability to bind to lipid membrane. In addition the number of phospholipid molecules per micelle can be systematically varied in a defined physical environment, the mixed micelle. On the other hand, the liposome assay where phospholipids are located in the bilayer vesicles provides a surface resembling the plasma membrane; thus, a more physiological environment for interaction of phospholipid and the enzyme. The regulation of PLC δ1 in vitro critically depends on phospholipid, polyamines, and Ca^{2+} [20,45,46]. Sphingomyelin is the most effective of the phospholipids tested for its ability to inhibit PLC δ1 [20]. Sphingosine, which is on the pathway of SM degradation, activates PLC δ1 moderately. The other homolog of sphingosine 4-hydroxysphinganine (phytosphingosine) activates PLC δ1 to a lesser extent (Fig. 1). Investigating the effect of sphingosine on PLC δ1 activity, we found that sphingosine in 0–50 μM range activates PLC δ1 in all assays employed.

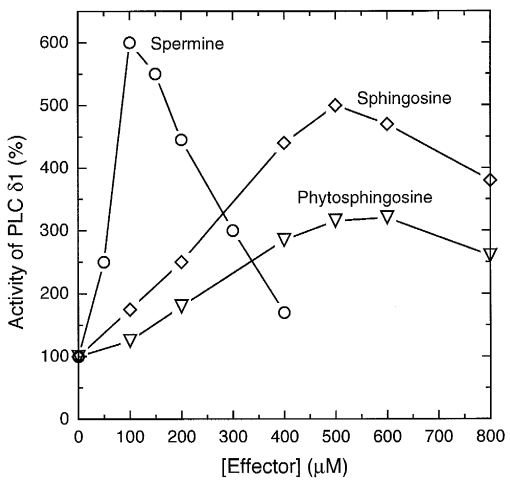

Fig. 1. Effect of sphingosine, phytosphingosine and spermine on the activity of PLC δ1. The detergent assay was used as described in Section 2. One hundred percent activity was 1.36 nmol/ml/min. The average standard deviation for all points was $\pm$3.7% (n = 4).

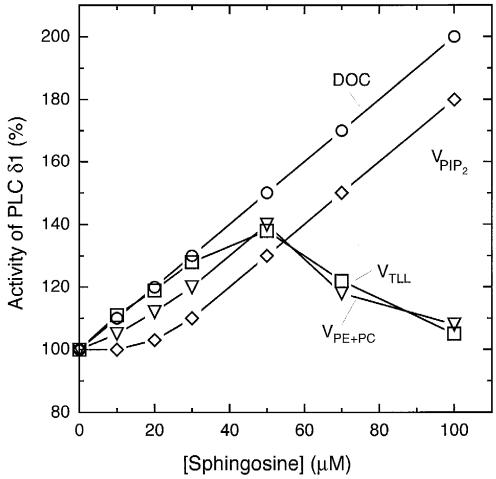

Fig. 2. Effect of sphingosine on PLC δ1 activity in different assays. The reaction mixture for each assay was as described in Section 2. DOC, the sodium deoxycholate assay. V_{PIP2}, the liposome assay, the vesicles consisted PIP_2 (25 nmol/ml) only. V_{TLL}, liposome assay, the vesicles consisted 200 nmol/ml total liver lipids, 25 nmol/ml PIP_2. V_{PE+PC}, liposome assay, the vesicles consisted 200 nmol/ml PE + PC (9:1) and 25 nmol/ml PIP_2. 100% represents activities of 0.97 (DOC), 0.82 (V_{PIP2}), 0.93 (V_{TLL}) and 0.75 (V_{PE+PC}) nmol PIP_2 hydrolyzed/ml/min. The average standard deviation for all points was; DOC, $\pm$3.9% (n = 5); V_{PIP2}, $\pm$5.4% (n = 3); V_{TLL}, $\pm$6.7% (n = 4), V_{PE+PC}, $\pm$7.5% (n = 3).

However, in the liposome assay activation of PLC δ1 by sphingosine declined when sphingosine concentration increased above 50 μM (molar ratio phospholipids : sphingosine 4:1) (Fig. 2). The simplest explanation of this observation could be the substrate redistribution between inner and outer leaflet of the phospholipid bilayer caused by sphingosine. In order to examine such a possibility we have allowed the liposome assay to continue. The liposome (TLL) assay leveled off when about 38% of the 25 nmol/ml of PIP_2 was hydrolyzed either in the presence or absence of sphingosine at concentrations of 50–200 μM. Upon adding 2.4 mM deoxycholate to the reaction mixture, the remainder of the substrate was hydrolyzed to within 98–99%. Effect of 50 μM sphingosine on accessibility and time course of PIP_2 hydrolysis in TLL liposome assay is shown in Fig. 3. Based on the results obtained, we conclude that the distribution of PIP_2 between the two membrane leaflets of TLL liposomes was not affected by sphingosine (50–200 μM). The same effect of sphingosine

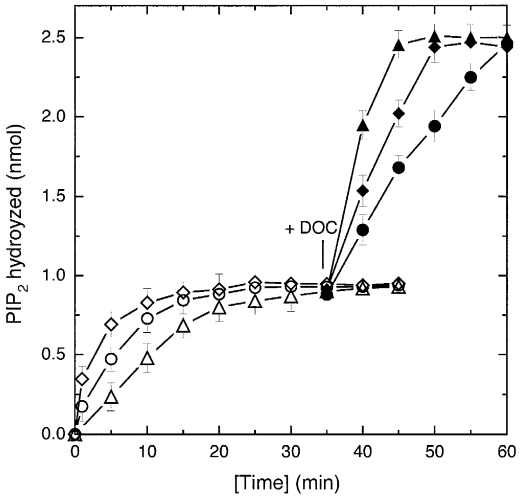

Fig. 3. Effect of sphingosine on the distribution of PIP_2 in TLL liposomes. The reaction mixture was as described in Section 2. The vesicles consisted 200 nmol/ml of total liver lipids and 25 nmol/ml PIP_2. The concentration of sphingosine was 50 nmol/ml (◇), 200 nmol/ml (△). In the control sphingosine was omitted (o). At 35 min 50% of the reaction mixture was withdrawn and to this sample deoxycholate was added to concentration of 2.4 mM (closed symbols). Each point represents the mean ± S.D. of triplicate determinations.

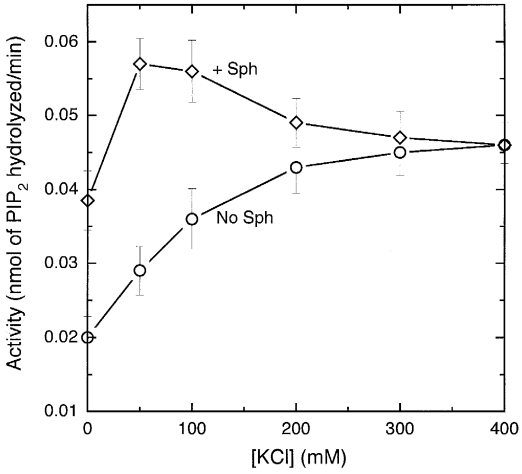

Fig. 4. Effect of KCl concentration on PLC δ1 activity in the liposome (PE + PC) assay in the presence of sphingosine. The reaction mixture was as described in Section 2. The vesicles consisted 200 nmol/ml of PE + PC (9:1) and 25 nmol/ml PIP_2. The sphingosine (Sph) concentration was 50 nmol/ml. Each point represents the mean ± S.D. ($n = 3$)

was observed in assay with PE + PC liposomes. However, the distribution of PIP_2 between the two membrane leaflets of PE + PC liposomes was different from that of TLL liposomes. In PE + PC liposomes 65% of PIP_2 faces outward (not showed). Data from experiments with detergent and liposome assay suggested that the nature of sphingosine (up to 50 μM) interaction with PLC δ1 is the same in both kinds of assay. Therefore in our experiments we chose to use sphingosine at concentrations up to 50 μM that is close to concentration of this agent in living cells [47–49]. In interaction of sphingosine with PLC δ1, ionic as well as hydrophobic forces may be involved. In the liposome assay, the activity of PLC δ1 increased with the increasing KCl concentration (Fig. 4). These results may indicate that for interaction of the enzyme with PIP_2 located in the phospholipid membrane hydrophobic forces are involved. Increasing ionic strength results in a weakening of the electrostatic intermolecular interactions, allowing hydrophobic interactions to take place. The highest activation of PLC δ1 by sphingosine occurs at 50 mM KCl and it diminishes with increasing KCl (Fig. 4). Increase in ionic strength of the reaction medium from 0.08 to 0.4 M caused a decrease in activation of PLC δ1 by sphingosine from 200% to 0%. These results may indicate that in interaction of PLC δ1 with sphingosine electrostatic forces are involved and that PIP_2 and sphingosine interact with different protein domains of the enzyme. This assumption was confirmed by the experiments with the

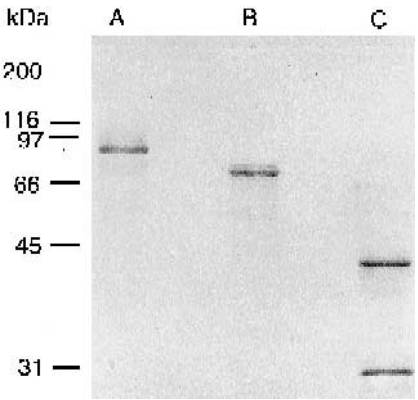

Fig. 5. Limited proteolysis of rat liver PLC δ1 by trypsin. Rat liver PLC δ1 was incubated with trypsin as described in Section 2. The active fragments were purified by ion exchange chromatography and subjected to SDS-PAGE on 12% gel. Intact rat liver PLC δ1 (lane 1); purified active fragment of PLC δ1 obtained by 15 min (lane 2) and 1 h (lane 3) incubation with trypsin.

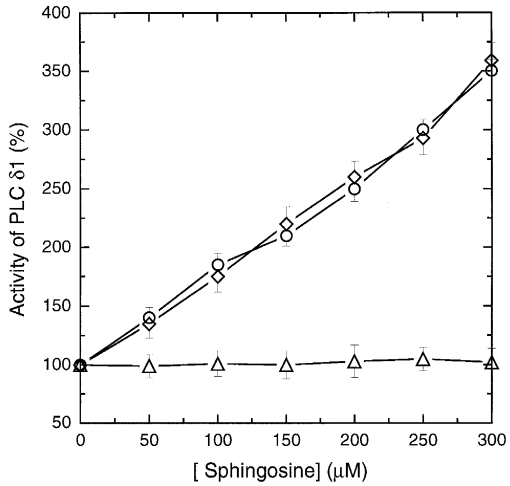

Fig. 6. The effect of sphingosine on the activity of PLC d1 and its proteolytic fragments. The detergent assay was used as described in Section 2. Activity of the native PLC d1 (o), the 77 kDa fragment (◇), the 70 kDa fragment (△). One hundred percent activity for native PLC d1 was 1.36 nmol/ml/min, for 77 kDa fragment was 0.2 nmol/ml/min, for 70 kDa fragment was 0.18 nmol/ml/min. The results are mean values ±S.D. ($n = 3$).

active fragments of PLC $\delta 1$ generated by proteolysis with trypsin. Under employed conditions we have obtained two kinds of PLC $\delta 1$ active fragment. Limited proteolysis of PLC $\delta 1$ with trypsin for 15 min resulted in the generation of a catalytically active 77 kDa fragment (Fig. 5). Sequence analysis reveals that trypsin digestion of PLC $\delta 1$ resulted in the generation of 77 kDa fragment, which lacked the first 60 amino acids. The N-terminal part of PLC $\delta 1$ within first 135 amino acids contains a pleckstrin homology (PH) domain, which is required for membrane attachment [50,51] and PIP_2 [52] and PI_3 binding [53]. It was reported that 77 kDa fragment of PLC $\delta 1$ does not bind to phospholipid vesicles containing PIP_2 [50,53], thus is inactive in liposome assay. In detergent assay the effect of sphingosine on intact PLC $\delta 1$ and 77 kDa fragment was the same (Fig. 6). This may indicate that interaction of sphingosine with PH domain of PLC $\delta 1$ is not necessary for activation of the enzyme. Previously it was reported that prolonged digestion of PLC $\delta 1$ with trypsin generates smaller active fragments of the enzyme [50,54,55]. After 1 h digestion of PLC $\delta 1$ with trypsin and purification on Mono S cation exchange column, we obtained a 70 kDa catalytically active product which on SDS-PAGE separates into 40 kDa and 30 kDa polypeptides (Fig. 5). Sequencing of the generated polypeptides confirmed sequences reported previously and showed that the 40 kDa polypeptide contains the aminoterminal part of region X and the 30 kDa polypeptide corresponded to part of the spanning region and complete region Y [54]. Data from our experiments with 70 kDa PLC $\delta 1$ (not shown) are very similar to those obtained by other in respect to activation by calcium, rate of PIP_2 hydrolysis in detergent assay and lack of binding to phospholipid vesicles [50,54]. As can be seen in Fig. 6 sphingosine does not activate the 70 kDa PLC $\delta 1$ in detergent assay. This may indicate that in interaction of sphingosine with PLC $\delta 1$, intact region spanning X and Y is necessary.

Because sphingosine is an metabolite of sphingomyelin we tested the effect of sphingosine on inhibition of PLC $\delta 1$ caused by SM. Fig. 7 illustrates the effect of sphingosine and sphingomyelin on PLC $\delta 1$ activity measured in liposome (TLL) assay. The presence of 50 μM sphingosine protects PLC $\delta 1$ against inhibition caused by 50 μM sphingomyelin.

In the action of PLC $\delta 1$ on the substrate localized in cell membrane two main steps can be distinguished, i.e., binding to the membrane surface and

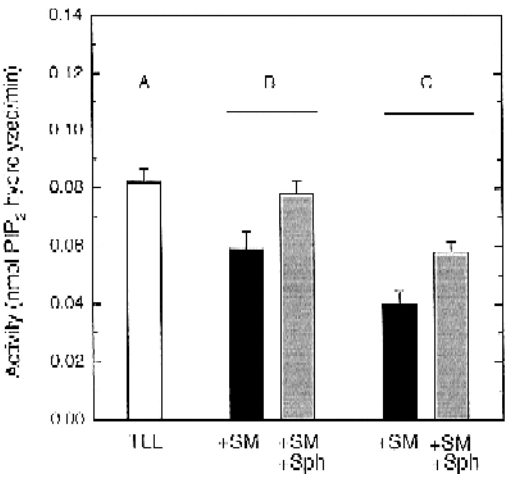

Fig. 7. Effect of sphingosine on the inhibition of PLC $\delta 1$ by sphingomyelin in the liposome assay. The reaction mixture was as described in Section 2. The vesicles consisted 200 nmol/ml of total liver lipids (TLL) and 25 nmo/mL PIP_2. A, TLL liposomes only. B, sphingomyelin (SM) 50 nmol/ml, sphingosine (Sph) 50 nmol/ml. C, sphingomyelin (SM) 100 nmol/ml, sphingosine (Sph) 50 nmol/ml. The results are mean values ±S.D. ($n = 4$)

interaction with the substrate. Beside lipid composition of the membrane, both steps of PLC δ1 action depend on the physical state of the membrane. Previously it was reported that the activity of PLC δ1 [56] and other PLC's [57,58] depends on surface pressure and the electrostatic surface potential of lipid membrane. Binding of several other proteins to phospholipid membrane decreases as the surface pressure increases [59–61]. The surface stress of lipid vesicles depends on lipid composition and the curvature of the membrane. In an attempt to set the assay of PLC δ1 in a more physiological context (in terms of physical-chemical properties of the membrane), we tested the effect of sphingosine in assay with erythrocyte membranes. We chose to use erythrocyte membrane because is the best characterized and simplest cell membrane widely used by other investigators. In erythrocytes incubated with [^{32}P]Pi, the only labeled lipids are polyphosphoinositides and phosphatidic acid [62]. This is because in these simple cells there is no synthesis of phosphatidylinositol (PI), but only a turnover of the 4-phosphate of PIP and of the 4- and 5-phosphate groups of PIP_2 by specific kinases and phosphatases and synthesis of PA from diacylglycerol by a specific kinase [63]. We used alkali-stripped inside-out vesicles containing labeled [^{32}P]phospholipids. We found that erythrocyte membrane assay leveled off when about 70–75% of total lipid ^{32}P was liberated as a water soluble compounds (not showed). This accounted for 92–97% of both PIP and PIP_2. The remaining 25–30% radioactivity was phosphatidic acid (see Ref. [63]). Assay conditions were chosen so that the 10–15% of the PIP + PIP_2 in the membranes were hydrolyzed. The data shown in Fig. 8 demonstrate that sphingosine in assay with erythrocyte membranes strongly inhibits activity of PLC δ1. Phytosphingosine (4-hydroxysphinganine), which is a homolog of sphingosine, inhibits PLC δ1 to much lesser extent. The concentrations for 50% inhibition by sphingosine and phytosphingosine were 15 and 50 nmol/ml, respectively. Palmitoyl carnitine at concentrations up to 100 nmol/ml did not inhibit PLC δ1 under conditions where sphingosine inhibited strongly (Fig. 8). Carnitine esters, and to the some extent sphingosine, have detergent-like properties. The observation that palmitoyl carnitine did not affect PLC δ1 activity indicates that the inhibition of the enzyme by sphingosine in the erythrocyte membrane assay is

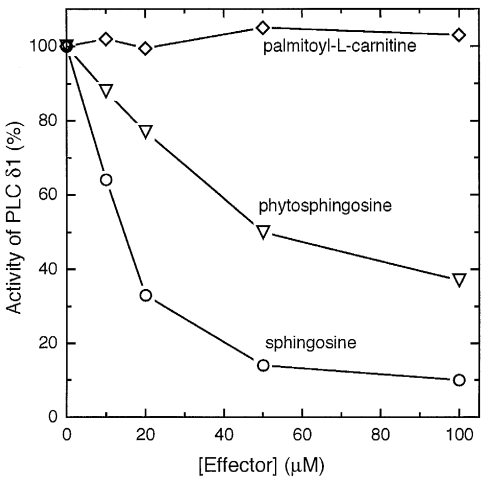

Fig. 8. Inhibition of PLC δ1 by sphingosine in the erythrocyte membrane assay. The reaction mixture was as described in Section 2. The reaction mixture contained 100 nmol/ml of erythrocyte membrane phospholipids (1300 cpm/nmol) and the effector at indicated concentrations. Total PIP_2 + PIP was 9620 cpm/assay. 100% represents an activity which releases to H_2O phase 925 cpm/5 min. The average standard deviation for all points was; sphingosine, ±10.6% ($n = 4$); phytosphingosine, ±9.2% ($n = 3$); palmitoyl-L-carnitine, ±7.3% ($n = 3$).

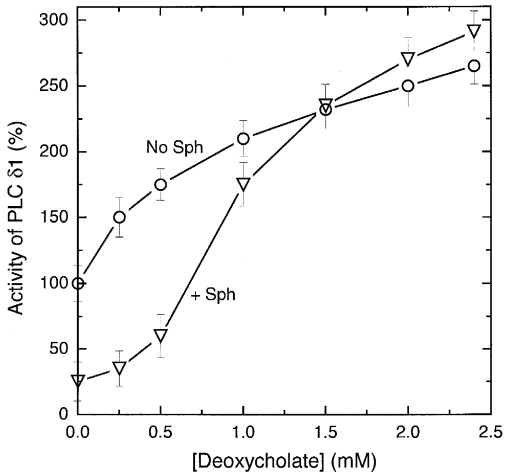

Fig. 9. Effect of sodium deoxycholate on the activity of PLC δ1 in the erythrocyte membrane assay. The reaction mixture was as described in Section 2. The reaction mixture contained 100 nmol/ml of erythrocyte membrane phospholipids (1400 cpm/nmol), sodium deoxycholate at indicated concentrations and 50 nmol/ml of sphingosine where indicated. Total PIP_2 + PIP was 10 500 cpm/assay. 100% represents an activity which releases to H_2O phase 565 cpm/5 min. The results are mean values ±S.D. ($n = 3$).

not due to a non-specific detergent effect. On the other hand, in the presence of increasing concentrations of sodium deoxycholate, the inhibition of PLC δ1 by sphingosine declined (Fig. 9). Inhibition of PLC δ1 by sphingosine was completely abolished by 1.5 mM deoxycholate.

4. Discussion

The inhibitory effect of sphingomyelin on PLC δ1 activity was described previously [20]. In binding experiments PLC δ1 shows a high degree of specificity for PIP_2 and sphingomyelin [21,22]. It has been proposed that, in vivo, sphingomyelin acts as an inhibitor of PLC δ1, which enables the enzyme to be regulated by activation [20]. A number of extracellular signaling molecules can induce turnover and transient decline of sphingomyelin [64,65], possibly leading to a partial relief of PLC δ1 inhibition. Here, we showed that sphingosine, which is on the pathway of sphingomyelin degradation, activates PLC δ1 moderately in the liposome and detergent assay (Figs. 1 and 2). However, in the assay with erythrocyte membranes sphingosine becomes a strong inhibitor of the enzyme (Fig. 8). The stimulatory effect of sphingosine on PLC δ1 activity in the detergent assay and the assay with substrate as a small liposome formed from PIP_2 (Fig. 2) indicates that sphingosine does not interfere with binding of the enzyme to its substrate. The weak activation of PLC δ1 observed in both assays seems to be the effect of structural changes of the enzyme induced by sphingosine binding. Increased ionic strength of the reaction medium (0.04–0.2 M) in the liposome assay resulted in activation of PLC δ1 to the same extent as 50 nmol/ml sphingosine (Fig. 4). The structural changes of PLC δ1 induced by binding of sphingosine may affect interaction of the enzyme with other phospholipids. In the assay with liposome composed of PE + PC and liposomes containing TLL sphingosine at concentration higher than 50 nmol/ml does not activate PLC δ1, but instead becomes an inhibitor of the enzyme (Fig. 2). Thus, diminution of the sphingomyelin inhibition of PLC δ1 observed in the presence of sphingosine in the liposome assay may be explained by the decreased binding of SM to the enzyme resulting from changes in the protein structure (Fig. 5). Results obtained from experiments with the active fragments of PLC δ1 indicate that for interaction of sphingosine with the enzyme, intact regions that span domain X and Y is necessary (Fig. 6). In rat PLC δ1 this spanning region contains 49 residues with a high percentage of acidic amino acids [66]. Between residues 450 and 472, seven glutamic acids and three aspartic acids are located. It might be assumed that interaction of polar lipids especially with positively charged head group takes place within this region of PLC δ1. The exact mechanism by which sphingosine inhibits PLC δ1 in the assay with erythrocyte membranes remains undefined. The possibility exists that sphingosine inhibits activity of PLC δ1 by competition in interaction of the enzyme with the positively charged lipids located in the cell membrane. Alternatively, it might be assumed that sphingosine is involved in the generation of binding sites within erythrocyte membrane which exclude the enzyme from the substrate. The addition of the deoxycholate prevents such structures from occurring and restores the PLC δ1 activity (Fig. 9). The presented results are suggestive of sphingosine being the physiological inhibitor of PLC δ1 if it is assumed that the experiments with erythrocyte membranes resemble in vivo conditions.

Agonist-stimulated breakdown of sphingomyelin results in ceramide generation which may be converted to sphingosine by the action of neutral or acid ceramidase [64,65,67]. Assuming that, under physiological conditions, PLC δ1 is bound to sphingomyelin in cellular membranes in an inactive form, then it may be speculated that hydrolysis of SM should result in activation of PLC δ1, but vitamin D_3 or TNF-α-stimulated hydrolysis of SM did not induce accumulation of DAG or IP_3 [69,70]. The lack of expected alteration in phosphoinositide breakdown under declined level of SM may possibly be explained by an inhibitory effect of sphingosine on PLC δ1 or/and by involvement of other regulatory factors substantial for controlling this enzyme activity. Wilson and colleagues [47] demonstrated that sphingomyelinase-generated sphingosine is regulated by external effectors and that the levels of free sphingosine in the human neutrophils cells can reach 7 nmol per gram of wet tissue [48]. On the other hand it has been reported that no increase in sphingosine could be detected in response to the action of 1,25-$(OH)_2D_3$

on HL-60 cells [71]. In human A431 epidermoid carcinoma cells, the addition of 0.1–1 μM sphingosine to the incubation medium resulted in an increased cellular ceramide level while the sphingosine level was not raised [72]. Although, at sphingosine levels higher than 1 μM in the incubation medium the cellular level of ceramide did not increase further, while intracellular sphingosine was extremely elevated [72]. Reported cellular sphingosine content varied depending on cell type and quantitation method. The sphingosine content of rodent liver is 7–14 nmol/g fresh wt [33,48,49]. Half of this is bound to the plasma membrane fraction. Incubation of isolated plasma membrane results in about a 3-fold rise in membrane-bound sphingosine [68]. Granulocytes contain about 16 nmol sphingosine/ml packed cells [47]. Assuming that 50% of the packed cell volume is intracellular water, this yields a concentration of about 32 nmol/ml, which is at the high end of sphingosine concentrations. This is about two times greater than the concentration for 50% inhibition of PLC δ1 by sphingosine in the erythrocyte membrane assay.

Whatever the precise mechanism, our results raise the important question of whether cellular sphingosine, formed by degradation of sphingolipids acts to regulate the PLC δ1 activity. However, further experiments are required to define the mechanism by which sphingosine interacts with PLC δ1 acting on plasma membranes.

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