Regulation of inositol lipid-specific phospholipase Cδ by changes in Ca^{2+} ion concentrations

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Studies of inositol lipid-specific phospholipase C (PLC) have elucidated the main regulatory pathways for PLCβ and PLCγ but the regulation of PLCδ isoenzymes still remains obscure. Here we demonstrate that an increase in Ca^{2+} ion concentration within the physiological range (0.1–10 μM) is sufficient to stimulate PLCδ1, but not PLCγ1 and PLCβ1, to hydrolyse cellular inositol lipids present in permeabilized cells. The activity of PLCδ1 is further enhanced in the presence of phosphatidylinositol transfer protein (PI-TP). Both full activation by Ca^{2+} ions and stimulation in the presence of PI-TP require an intact PH domain involved in the membrane attachment of PLCδ1. The physiological implication of this study is that PLCδ1 could correspond to a previously uncharacterized PLC responsible for Ca^{2+} ion-stimulated inositol lipid hydrolysis observed in many cellular systems.

INTRODUCTION

A large number of extracellular signals stimulate the hydrolysis of PtdIns(4,5)P_2 by inositol lipid-specific phospholipase C (PLC). For the regulation of cellular processes, the best documented consequence of this hydrolysis is the generation of two second messengers, Ins(1,4,5)P_3 and diacylglycerol (DG), involved in the release of Ca^{2+} ions from intracellular stores and the stimulation of protein kinase C isoenzymes [1]. In addition to the second messenger production, the regulation of PtdIns(4,5)P_2 concentration itself could be relevant for cell signalling because many proteins bind and/or require PtdIns(4,5)P_2 to function [2,3].

The need to control the hydrolysis of PtdIns(4,5)P_2 stringently is reflected by the fact that there are many PLC isoenzymes and multiple mechanisms linking these isoenzymes to various receptors [2,4]. The PLCβ isoenzymes are activated through interaction with the α subunits of the pertussis toxin-insensitive G_q family of heterotrimeric G-proteins. The G-protein-coupled receptors that are known to utilize this $G_q\alpha$/PLCβ pathway include those for bradykinin, bombesin, angiotensin, histamine and vasopressin as well as muscarinic (m1, m2 and m3) and α1 adrenergic agonists. PLCβ isoenzymes are also activated by the $\beta\gamma$ subunits of pertussis toxin-sensitive G-proteins from G_i/G_o family. The m2 and m4 muscarinic acetylcholine receptors, fMetLeuPhe receptor and interleukin 8 receptor seem to be linked to the G$\beta\gamma$/PLCβ pathway. Members of PLCγ family are activated through interaction with and phosphorylation by receptor and non-receptor protein tyrosine kinases. Agonists for receptor tyrosine kinases such as platelet-derived growth factor, epidermal growth factor, fibroblast growth factor and nerve growth factor are known to stimulate PLCγ in a wide variety of cells. Non-receptor protein tyrosine kinases from several families are likely to provide a link between different receptors and PLCγ isoenzymes; examples include T-cell antigen, leukaemia inhibitory factor and angiotensin II receptors.

In comparison with PLCβ and PLCγ isoenzymes, the physiological role and regulation of members of PLCδ family have remained poorly defined. There are four PLCδ isoenzymes in mammalian cells (PLCδ1 to PLCδ4) and related enzymes have been isolated from yeast, slime mould and some plant species; PLCδ-like enzymes are the only type of PLC found in those organisms [5]. The use of genetic manipulations in yeast and slime mould revealed that the deletion of the PLCδ gene caused pleiotropic changes of the phenotype [6,7] or, possibly, their suppression [8]. Therefore a link between PLCδ and any of the signalling pathways described in those organisms could not be established. Studies in mammalian cells focused on PLCδ1 isoenzyme and, to a smaller extent, on recently described PLCδ4. It has been shown [9] that expression of PLCδ4 occurred at S-phase of the cell cycle and the enzyme was predominantly found in the nucleus. In contrast, PLCδ1 was not localized in nuclei and its expression was independent of the cell cycle. Findings that PLCδ1 could bind to p122 protein with Rho GAP activity [10] and a novel type of a GTP-binding protein Gh [11] suggested that its activity might be modulated through protein–protein interactions. However, studies of this isoenzyme also revealed that substances such as Ca^{2+} ions [12] and Ins(1,4,5)P_3 [13,14] could have an important regulatory role. Furthermore, structural studies of the multidomain PLCδ1 protein suggested binding sites for Ca^{2+} ions and the headgroup of PtdIns(4,5)P_2, both within and outside the catalytic domain [15,16]. Binding sites for non-catalytic Ca^{2+} ions have been described within the C2 domain and, possibly, the EF-hand domain, whereas the PH domain contains a binding site for the headgroup of PtdIns(4,5)P_2 and Ins(1,4,5)P_3. Comparisons of PLC sequences have shown that these ligand-binding sites found in PLCδ1 are not strictly conserved in the corresponding structural domains (PH, EF-hand and C2 domain) of PLCβ and PLCγ isoenzymes. Therefore interactions of PLCδ1 with Ca^{2+} ions or Ins(1,4,5)P_3 could underlie regulatory mechanisms that might be unique to this and some other PLCδ isoenzymes.

In this study we analysed the effect of Ca^{2+} ion concentration on the activities of different PLC isoenzymes towards the substrate present in permeabilized cells and found that changes in Ca^{2+} ion concentration within a physiological range selectively

Abbreviations used: CHO, Chinese hamster ovary; DG, diacylglycerol; GTP[S], guanosine 5′[γ-thio]triphosphate; PI-TP, phosphatidylinositol transfer protein; PLC, phospholipase C

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stimulated the activity of PLCδ1. This PLC isoenzyme could therefore mediate the well-documented stimulation of inositol lipid hydrolysis by an increase in intracellular Ca^{2+} ion concentration.

EXPERIMENTAL

Transient transfection of COS cells and preparation of cellular extracts

The cDNA species encoding rat brain PLCδ1, bovine brain PLCβ1 and bovine brain PLCγ1 were subcloned into eukaryotic expression vector pMT2 [17]. For transient transfection, COS 1 cells were incubated with plasmid DNA–lipofectAMINE (Gibco-Life Science) complex (0.7 μg/5 μl) for 6 h in serum-free medium and then grown for 30 h in Dulbecco's modified Eagle's medium/10 % (v/v) foetal bovine serum. The efficiency of transfection was between 30 % and 50 %. After being harvested, the cell pellets were resuspended in an equal volume of buffer A [20 mM Na-Pipes (pH 6.8)/137 mM NaCl/3 mM KCl] supplemented with a cocktail of complete protease inhibitors (Boehringer). The resuspended cells were then sonicated (Soniprep 150 at setting 14, twice for 5 s each) and subjected to centrifugation at 16000 ***g*** at 4 °C for 15 min. The resulting supernatant was applied to a PC 3.3/10 Fast Desalting column (Pharmacia) equilibrated with buffer A, and protein was eluted with the same buffer. PLC activity in these preparations was determined in a PtdIns(4,5)P_2/sodium cholate mixed-micelle assay (see below); calculated specific activities were 3.4 units/mg for PLCβ1, 1.6 units/mg for PLCγ1 and 3.2 units/mg for PLCδ1, similar to previously published values [17]. Enzyme units are defined in the section on Analysis of PLC by activity measurements and Western blotting. In accordance with these measurements the samples were adjusted to the same enzyme concentrations. For the reconstitution experiments with the permeabilized cells, aliquots containing 1–5 units of PLC activity were used.

Expression and purification of recombinant proteins

A bacterial expression vector pGEX-2T was used to generate PLCδ1 fusion proteins encoding the full-length PLCδ1, the protein lacking the first 132 amino acid residues (ΔPH-PLCδ1) and protein containing only the N-terminal part (residues 1–175) of PLCδ1 (PLCδ1PH domain) [18]. After the removal of glutathione S-transferase by cleavage with thrombin, all PLCδ1 proteins were purified as previously described [18] and the buffer was exchanged for buffer A on a Fast Desalting column. The specific activity of PLCδ1 preparations (in the range 800–1000 units/mg) was as described previously [18].

The preparation of the first C2 domain from synaptotagmin I as a glutathione S-transferase fusion protein was as described by Davletov and Südhof [19]. Before reconstitution with permeabilized cells the buffer was exchanged for buffer A on a Fast Desalting column.

The expression of phosphatidylinositol transfer protein α (PI-TPα) by using vector pET21α and protein purification on Ni^{2+}-nitrilotriacetic acid agarose was as described previously [20]. Unless otherwise stated, the concentration of PLCδ1 used for reconstitution experiments was 1–10 μg/ml and that of PI-TPα was 50–200 μg/ml.

Preparation of permeabilized cells and analysis of inositol lipid hydrolysis by PLCs

Labelling of inositol lipids in HL-60 cells with *myo*-[^{3}H]inositol (Amersham), cell permeabilization and the analysis of inositol lipid hydrolysis after addition of the required components were performed by the method of Cockcroft et al. [21]. Briefly, the labelled cells were permeabilized with Streptolysin O for 40 min at 37 °C (to deplete the cells of endogenous proteins including PLCs) and resuspended in a buffer A supplemented with 2 mM $MgCl_2$, 1 mM $MgATP^{2-}$, 10 mM LiCl, 1 mg/ml glucose and 1 mg/ml albumin. An aliquot of permeabilized cells was combined with different protein preparations in the supplemented buffer A and concentrations of free Ca^{2+} ions in the resulting reaction mixtures were maintained with Ca/EGTA buffers. When specified, 0.1–100 μM Ins(1,4,5)P_3 (Sigma), 10 μM guanosine 5′-[γ-thio]triphosphate (GTP[S]) (Boehringer), 0.5 mM dGDP or 2 mM 2,3-diphosphoglycerate (Sigma) was included in the assay mixture. Incubation was for 20 min at 37 °C, the reaction was quenched with chloroform/methanol (1:1, v/v) and the phases were separated by centrifugation. The upper phase was applied to a Dowex column and inositol phosphates were eluted with 1 M ammonium formate/0.1 M formic acid. For the analysis of different inositol phosphates, step elution was performed with 0.2 M ammonium formate for InsP_1, 0.4 M for InsP_2 and 1 M for InsP_3. Radioactivity in all fractions was determined by liquid-scintillation counting. The lower phase was used for inositol lipid separation by TLC [22], and the radioactivity in PtdIns, PtdInsP_1 and PtdInsP_2 was analysed with a PhosphorImager (Fujix BAS 1000).

Preparations of permeabilized PC12 cells [grown in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) horse serum and 5 % (v/v) foetal bovine serum] were prepared as described for HL-60 cells except that the concentration of *myo*-[^{3}H]inositol was increased from 1 to 2 μCi/ml, and it was added directly to the growth medium.

Analysis of PLC by activity measurements and Western blotting

The assay of hydrolysis of PtdIns(4,5)P_2 with the substrate/sodium cholate mixed micelles was the same as described previously [17]. In this assay, 1 unit of PLC activity corresponds to the hydrolysis of 1 μmol of PtdIns(4,5)P_2/min. For analysis by Western blotting, mouse anti-(bovine PLCβ1), anti-(bovine PLCγ1) and anti-(bovine PLCδ1) (Upstate Biotechnology) were used and the proteins were detected with an enhanced chemiluminescence kit (Amersham).

RESULTS AND DISCUSSION

Stimulation of inositol lipid hydrolysis in permeabilized adrenal phaeochromocytoma (PC12) cells by Ca^{2+} ions

The Ca^{2+} ion dependence of PLC activity towards inositol lipids *in vitro* has been clearly established for all PLC isoenzymes [23]. However, the Ca^{2+} ion activation of PLC *in vivo* proved to be a more complex issue. Although in many cell types PLC activation occurred at resting Ca^{2+} ion concentrations (10–100 nM), it was possible to demonstrate that in some systems an increase in cellular Ca^{2+} ion concentration resulted in the stimulation of PLC activity [4,24]. To test whether changes in Ca^{2+} ion concentration could affect the activity of PLCδ1 towards the cellular substrate, we employed an assay system that used permeabilized cells. It has previously been shown that the permeabilization of different cell types with streptolysin O results in a loss of cytosolic components, including PLC enzymes, without the fragmentation of cellular membranes containing regulatory proteins and phospholipid substrate [21]. These properties of permeabilized cells proved suitable for the reconstitution of regulatory pathways of PLCβ and PLCγ [20]. For studies of PLCδ1, initial experiments were performed with PC12

Table 1 Stimulation of inositol lipid hydrolysis by Ca^{2+} in permeabilized PC12 cells

PC12 cells were acutely permeabilized or permeabilized for 40 min and then analysed for inositol phosphate production in the absence or presence of purified PLCδ1 (10 μg/ml) or PI-TPα (50 μg/ml) at different Ca^{2+} concentrations. The permeabilized cells were incubated for 20 min at 37 °C. The results are averages of duplicate incubations that did not vary by more than 10%. The results presented here are representative of four experiments.

			Inositol phosphates produced (d.p.m.)		
Permeabilization	Addition	$[Ca^{2+}]$ (M)...	10^{-8}	10^{-6}	10^{-5}
Acute	None		1870	5069	14144
40 min	None		985	1268	3537
40 min	PLCδ		1474	3139	7198
40 min	PI-TP		1118	1312	8139
40 min	PLCδ + PI-TP		2383	11799	15382

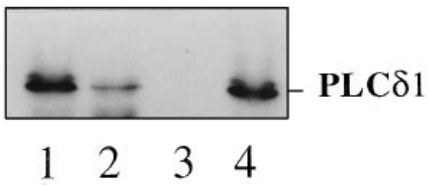

Figure 1 Release of PLCδ1 from PC12 cells after permeabilization

PC12 cells were prepared from six 75 cm^2 flasks and incubated in the presence of streptolysin O as described in the Experimental section. Samples prepared from cells permeabilized for 40 min (lanes 1 and 2) were compared with those prepared at time zero after the addition of streptolysin O (acutely permeabilized cells) (lanes 3 and 4). Amounts of PLCδ1 released from PC12 cells (in supernatants after sedimentation at 3000 ***g*** for 10 min) (lanes 1 and 3) and of PLCδ1 retained in cell pellets (lanes 2 and 4) were analysed. All samples were adjusted to equal volume, and aliquots (50 μl) were subjected to SDS/PAGE and Western blotting.

cells (Table 1), one of the few cell lines that express high levels of this isoenzyme [25]. Changes in Ca^{2+} ion concentration within the physiological range (10 nM to 10 μM) resulted in an increase of inositol lipid hydrolysis in acutely permeabilized cells, conditions under which the endogenous proteins were still present. Permeabilization of PC12 cells for prolonged periods (40 min) led to a loss of 90–95% of PLCδ1 protein from cells (Figure 1) and resulted in a decrease in responsiveness to Ca^{2+} ions (Table 1). The addition of purified PLCδ1 to these cells reconstituted higher levels of inositol lipid hydrolysis in the presence of 1 or 10 μM Ca^{2+} ions (Table 1). However, the inositol lipid hydrolysis did not reach levels observed in acutely permeabilized cells, suggesting that other cytosolic factors might participate in this process.

It has been shown that in permeabilized cells depleted of cytosol, the activities of reconstituted PLCβ [22], PLCγ [20] or inositol lipid kinases [26] were greatly enhanced by the addition of cytosolic PI-TP. Several PI-TPs have been isolated (PI-TPα and PI-TPβ in mammalian cells and SEC14 in yeast); they all have a similar stimulatory effect on PLC activity [20], functioning to increase the pool of PtdIns(4,5)P_2 accessible to the enzymes [22]. The addition of PI-TPα to permeabilized PC12 cells enhanced PLCδ1 activity and resulted in high levels of inositol lipid hydrolysis (similar to those seen in acutely permeabilized cells) when Ca^{2+} ion concentrations were increased from 10 nM to 1–10 μM (Table 1). The range of Ca^{2+} ion concentrations required for stimulation of PLCδ1 in the presence of PI-TP is comparable with the range of Ca^{2+} ion concentrations described for a stimulated cell.

Reconstitution experiments in permeabilized cells, similar to those suggesting the stimulation of PLCδ1 by Ca^{2+} ions in PC12 cells (Table 1), were performed on cell lines without detectable levels of this PLC isoenzyme. In human promyelocytic leukaemia (HL-60) cells, which have been previously used for the G-protein-mediated activation of PLCβ, the effect of Ca^{2+} ions on the activity of PLCδ1 added to the permeabilized cells could be clearly observed. An additional advantage of this cell line over PC12 cells was that after permeabilization the levels of inositol phosphates were not significantly affected by an increase in Ca^{2+} ion concentration or by PI-TP in the absence of added PLCδ1 (see Figure 5A). Therefore further studies of Ca^{2+} ion-stimulated inositol lipid hydrolysis, properties of PLCδ1 and PI-TP stimulation, were performed on permeabilized HL-60 cells.

Selective regulation of PLC isoenzymes by an increase in Ca^{2+} ion concentration

Although a number of studies suggested Ca^{2+} ion stimulation of PLC activity *in vivo* [4,24] it was not clear whether Ca^{2+} ions have a general stimulatory effect on all PLCs or stimulated selectively one of many mammalian isoenzymes. To address this critical question and clarify whether the observed Ca^{2+} ion effect (Table 1) was specific for PLCδ1, we compared isoenzymes from three PLC families (PLCδ1, PLCγ1 and PLCβ1) in a reconstitution assay with permeabilized HL-60 cells. For comparison of PLCδ1, PLCγ1 and PLCβ1 in this system, the enzymes were prepared from transiently transfected COS cells (Figure 2A). PLC activity in these preparations was first measured with PtdIns(4,5)P_2/sodium cholate mixed micelles; selected conditions and substrate presentation allowed measurements of PLC activity in the absence of upstream stimulatory components; it has been reported [23] that in this type of assay purified PLCδ1, PLCγ1 and PLCβ1 have similar specific activities. PLC activity in transfected cells was approx. 100-fold higher than in control COS cells. Analysis of PLC isoenzymes by Western blotting (Figure 2A, upper panel) showed that in comparison with transfected cells, amounts of PLC proteins in COS or HL-60 cells were negligible. The extracts from transfected COS cells, normalized for PLC activity in the mixed micelle assay, were added to permeabilized HL-60 cells and inositol phosphate production was measured at different Ca^{2+} ion concentrations within a physiological range (10 nM to 10 μM) (Figure 2A, lower panel). In this system an increase in Ca^{2+} ion concentration on its own was insufficient to activate PLCβ1 and PLCγ1 isoenzymes. In the PLCβ1 preparation a less pronounced effect of Ca^{2+} ions could be seen only after addition of GTP[S] to the reconstitution system with HL-60 cells, i.e. after activation by G-protein subunits (results not shown). These results are consistent with previous observations from analysis of purified PLCβ1 and PLCγ1 in permeabilized cells [20,22]. In contrast with other isoenzymes, the activity of PLCδ1 was approx. 10-fold higher when the Ca^{2+} ion concentration was increased to 1–10 μM (Figure 2A, lower panel). Preparations of PLCδ1 obtained after transient transfection of COS cells and of PLCδ1 overexpressed in bacteria and purified to apparent homogeneity showed comparable responses to an increase in Ca^{2+} ion concentration (results not shown). Thus the effect of an increase in Ca^{2+} ion concentration on PLC activity towards the substrate present in cellular membranes seems to be selective, and among the isoenzymes tested only PLCδ1 was activated in the absence of any additional stimuli.

At present the possibility cannot be excluded that the activation

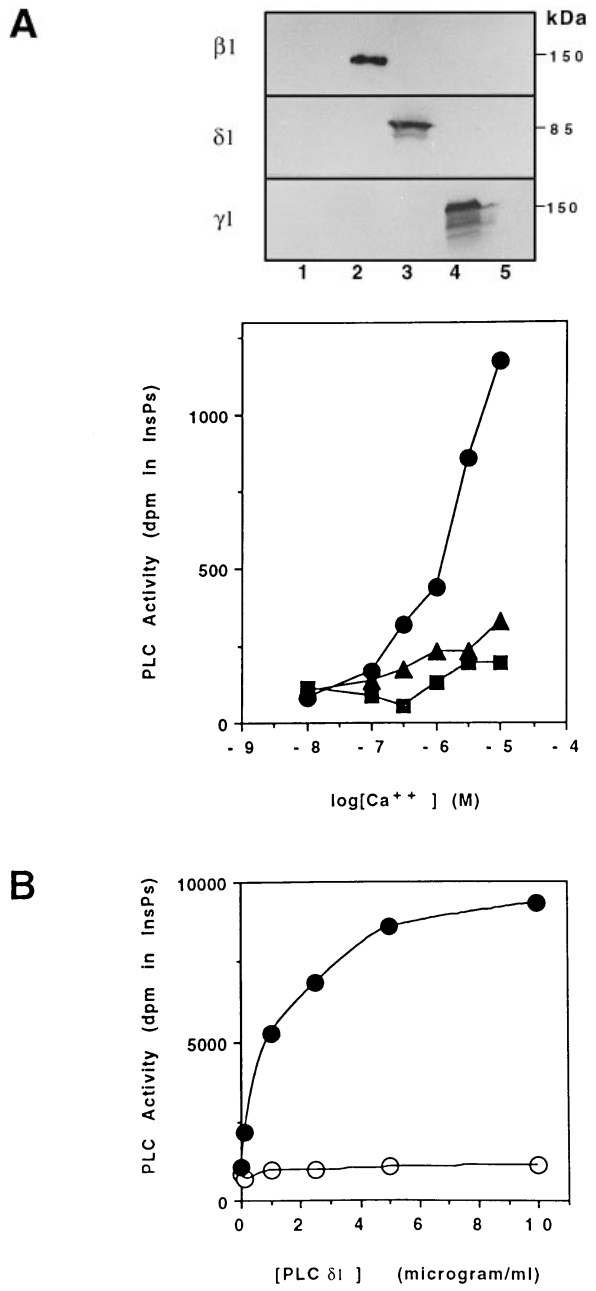

Figure 2 Inositol lipid hydrolysis in permeabilized HL-60 cells by PLCδ1, PLCβ1 and PLCγ1

(**A**) Expression levels of PLC isoenzymes were analysed by Western blotting (upper panel). Cellular extracts were prepared from control COS cells (lane 1), COS cells transfected with the plasmid encoding PLCβ1 (lane 2), PLCδ1 (lane 3) or PLCγ1 (lane 4), and HL-60 cells (lane 5). For measurements of inositol phosphate production over the range of Ca^{2+} ion concentrations (lower panel), extracts from transfected COS cells containing equal amounts of PLC activity, determined in the PtdIns(4,5)P_2 mixed-micelle assay, were added to permeabilized HL-60 cells. The extracts were from PLCβ1 (■), PLCδ1 (●) and PLCγ1 (▲) transfected cells. The permeabilized cells were incubated for 20 min at 37 °C. The data are averages of duplicate incubations that did not vary by more than 5%. The results presented here are representative of three experiments; similar data have been obtained with partly purified PLC preparations. (**B**) Increasing concentrations of purified PLCδ1 were analysed at 10 μM (●) and 10 nM (○) Ca^{2+} ions. The permeabilized cells were incubated for 20 min at 37 °C. The data are averages of duplicate incubations that did not vary by more than 5%. The experiment presented here is representative of four experiments.

of PLCδ1 by Ca^{2+} ions is an indirect effect and involves the binding of Ca^{2+} ions to another protein component present in the reconstitution system. However, findings that stimulation by Ca^{2+} ions can be achieved with purified PLCδ1 in different preparations of permeabilized cells, regardless of whether or not they express this isoenzyme, support a direct effect of Ca^{2+} ions. Furthermore PLCδ1 itself has several Ca^{2+} ion-binding sites, some of which could be specific to δ isoenzymes [5]. The EF-hand domain consists of four loop–helix–loop motifs in all PLCs but only one or two EF-hands in PLCδ isoenzymes show similarity with a canonical Ca^{2+} ion-binding motif found in other proteins. The possible function of this motif in the first EF-hand of PLCδ1 has been tested previously and it was found that mutations within this site did not abolish PLC stimulation by Ca^{2+} ion ionophore in transfected Chinese hamster ovary (CHO) cells [27]. The other Ca^{2+} ion-binding site that is clearly present in PLCδ1 but not strictly conserved in PLCβ and PLCγ isoenzymes is located within the C2 domain; a function of this site has yet to be analysed. The possibility also remains that Ca^{2+} ions could act through a common catalytic site that could be exposed in PLCδ1 but masked in unstimulated PLCβ and PLCγ isoenzymes.

Properties of Ca^{2+} ion-activated PLCδ1

In previous studies the characterization of PLCδ isoenzymes was mainly performed *in vitro* with inositol lipid/detergent mixed micelles as a substrate [6,23,28]. The activation of PLCδ1 by Ca^{2+} ions in the reconstitution system used here allowed studies to be made of the hydrolysis of cellular substrate present in permeabilized cell preparations.

The substrate hydrolysis in the presence of increasing concentrations of PLCδ1 protein reached approx. 50% of the maximum at 1 μg/ml (Figure 2B). This value is within the range of protein concentrations of PLCβ and PLCγ previously used to reconstitute their regulatory pathways in permeabilized cells [20,22]. A comparison of PLC activity over the range of protein concentrations at 10 nM and 10 μM Ca^{2+} ions demonstrated an absolute requirement for higher Ca^{2+} ion levels even at PLCδ1 concentrations of 100 μg/ml. This is consistent with observations that overexpression of PLCδ1 in COS cells [18] or CHO cells [12] did not lead to an increase in basal levels of inositol lipid hydrolysis. Similar data were reported for cells overexpressing PLCγ and PLCβ isoenzymes.

The substrate specificities of PLCδ isoenzymes have been previously studied in a mixed-micelle assay where PtdIns(4,5)P_2, PtdIns(4)P and PtdIns were analysed individually. These experiments have shown that PtdIns(4,5)P_2 and PtdIns(4)P were much better substrates than PtdIns at physiological Ca^{2+} ion concentrations [6,23,28]. Analysis of inositol phosphates produced by Ca^{2+} ion-activated PLCδ1 and analysis of the decrease in amounts of inositol lipids suggested that this preference was retained when the substrate was presented within permeabilized cells. The production of inositol trisphosphate and inositol bisphosphate was clearly detected after different times of incubation (1–30 min). A greater decrease in polyphosphoinositide amounts was observed at all time points; the decreases in amounts of inositol lipids, which could be more accurately measured at later time points (20 min), are illustrated in Figure 3.

Several reports have suggested that the activity of PLCδ can be modified by GTP-binding proteins [11,12]. In permeabilized HL-60 cells the addition of GTP[S], known to stimulate PLCβ1 and PLCβ2, or dGDP, with an inhibitory effect on these isoenzymes [21], did not have any effect on PLCδ1 activity (results not shown). Because the stimulatory effect on PLCβ isoenzymes is

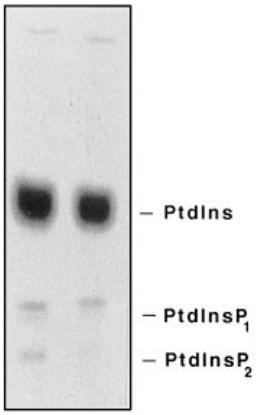

Figure 3 Changes in amounts of inositol lipids in permeabilized HL-60 cells after addition of PLCδ1

PtdIns, PtdInsP_1 and PtdInsP_2 were separated by TLC and analysed with a PhosphorImager. Samples were prepared from permeabilized HL-60 cells incubated for 20 min at 37 °C in the absence (left lane) or presence (right lane) of PLCδ1. The experiment was done in triplicate and a representative lane illustrating the changes in lipids is shown. Amounts of inositol lipids in the presence of PLCδ1 were expressed as a percentage of control levels; PI levels were decreased by 20%, PtdInsP_1 by 35% and PtdInsP_2 by 70%.

mediated through the activation of G_q and G_i proteins present in HL-60 cells, it is unlikely that subunits of these G-proteins ($G_q\alpha$ or $G\beta\gamma$) have a role in PLCδ1 stimulation. The same conclusion was reached from studies of G-protein regulation of PLC isoenzymes with different assay systems [29,30]. However, these results do not exclude a possibility that G-proteins present in some other cell types could have an effect on PLCδ1 activity.

Requirement for the PH domain

Structural studies of PLCδ1 [15] suggested that this enzyme could make multiple interactions with cellular membranes. These interactions could involve the PH domain, the hydrophobic ridge of the catalytic domain and the C2 domain (Figure 4A). Previous studies have also demonstrated an essential role for the PH domain in the interaction of PLCδ1 with the plasma membrane [18] and for the processive catalysis *in vitro* with mixed micelles of specific composition [31]. Analysis of PLCδ1 and the PH domain in isolation identified ligands for this domain as PtdIns(4,5)P_2 and Ins(1,4,5)P_3; it has been speculated that the binding of PtdIns(4,5)P_2 by the PH domain could be the critical interaction required for the localization of PLCδ1 to cellular membranes [32]. To analyse the importance of the PH domain for activity of PLCδ1 in the reconstitution system with permeabilized cells, we compared the full-length PLCδ1 with the deletion mutant lacking the PH domain (ΔPH-PLCδ1). When assayed in PtdIns(4,5)P_2/sodium cholate mixed micelles these proteins have similar specific activities [18], demonstrating that deletion of the PH domain does not affect the function of the active site. The PLC activities of the full-length and ΔPH-PLCδ1 in the assay with permeabilized HL-60 cells were markedly different; the activity of the Ca^{2+} ion-stimulated deletion mutant was decreased to approx. 1/40 (Figures 4B and 5C). Functions of the PH domain, most probably its ability to interact with cellular membranes, are therefore essential for the hydrolysis of cellular inositol lipids. It has been shown that the binding of PLCδ1 to PtdIns(4,5)P_2-containing vesicles *in vitro* did not require Ca^{2+} ions [33] and the plasma membrane attachment demonstrated in unstimulated cells [18]. Furthermore an increase in

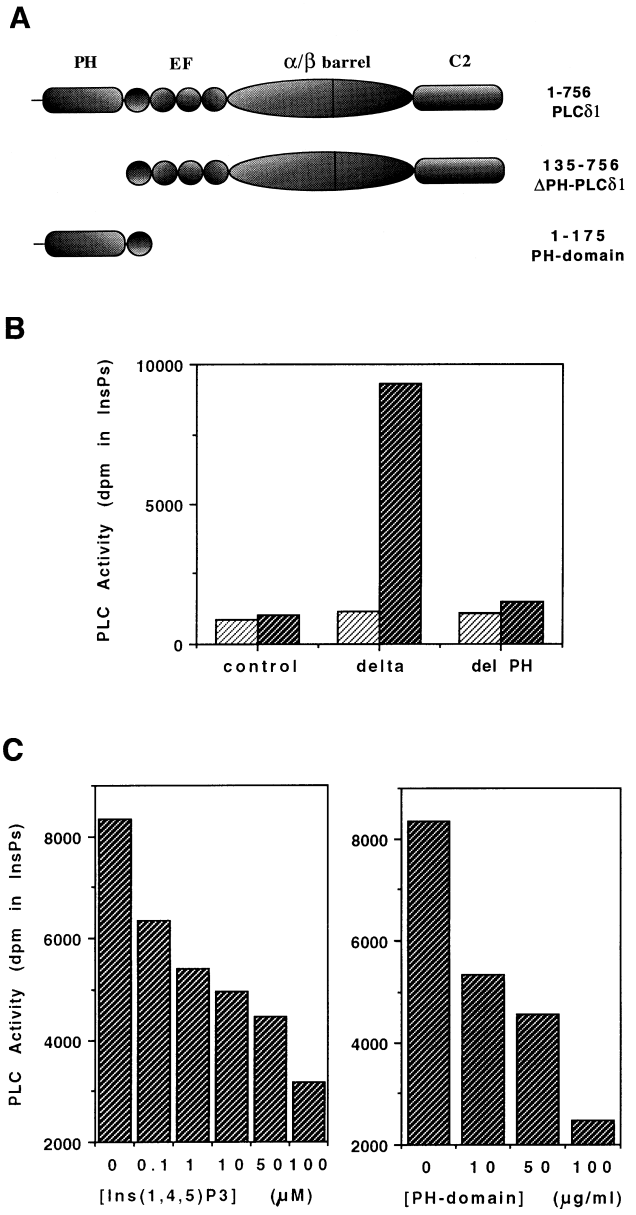

Figure 4 Effects of the PH domain deletion, the addition of Ins(1,4,5)P_3 and the PH domain protein on the activity of PLCδ1

(**A**) Schematic representation of the full-length PLCδ1 (containing the PH domain, the EF-hand domain, the catalytic α/β barrel and the C2 domain), the deletion mutant lacking the PH domain and the N-terminal construct containing the PH domain. (**B**) Inositol phosphate production in permeabilized HL-60 cells in the absence of other components (control) and in the presence of the full-length PLCδ1 (delta) or deletion mutant lacking the PH domain (del PH). The concentration of both proteins was 10 μg/ml. Ca^{2+} ion concentrations were 10 nM (light hatched) and 10 μM (dark hatched). The data are representative of four experiments performed in duplicate with the error within 10%. (**C**) The activity of the full-length PLCδ1 was analysed at 10 μM Ca^{2+} ions in the presence of increasing concentrations of Ins(1,4,5)P_3 (left panel) and the PH domain protein (right panel). The data are presented as in (**B**).

PLCδ1 protein in membrane fractions of transfected cells did not result in an increase in basal inositol lipid hydrolysis [12], suggesting that the Ca^{2+} ion-independent attachment could precede an activation step occurring only at higher Ca^{2+} ion concentrations.

ΔPH-PLCδ1 retained two other potential interaction sites with

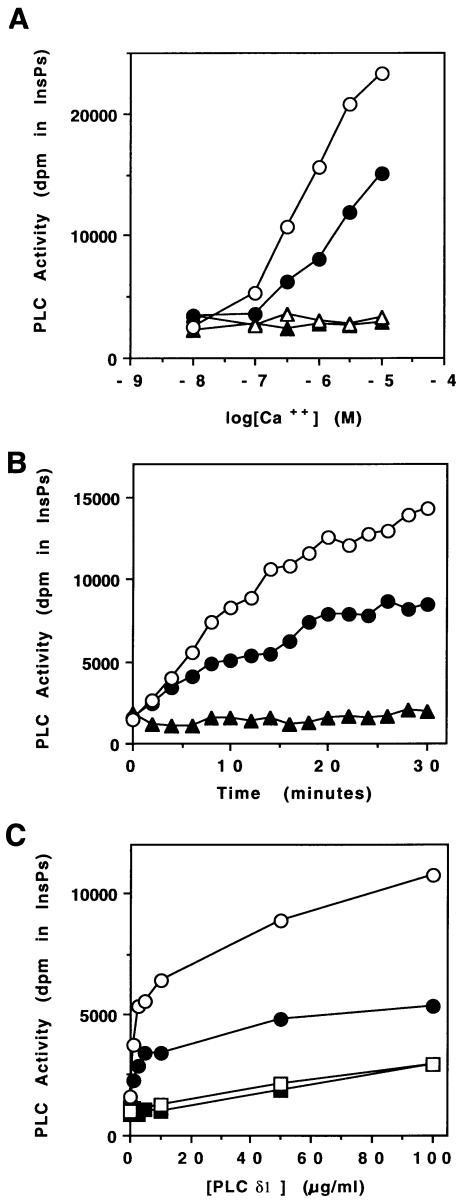

Figure 5 Effect of PI-TPα on activity of the full-length PLCδ1 and the PLCδ1 deletion mutant lacking the PH domain

(**A**) The production of inositol phosphates in permeabilized HL-60 cells over the range of Ca^{2+} ion concentrations was analysed in the absence of additional components (▲), in the presence of PLCδ1 (●), in the presence of PI-TPα (△) and in the presence of PLCδ1 and PI-TPα (○). The concentration of PLCδ1 was 10 μg/ml and that of PI-TPα was 100 μg/ml. (**B**) The production of inositol phosphates after different incubation periods was analysed in the absence of additional components (▲), in the presence of PLCδ1 (●) and in the presence of PLCδ1 and PI-TPα (○). The Ca^{2+} ion concentration was 10 μM. The concentration of PLCδ1 was 10 μg/ml and that of PI-TPα was 100 μg/ml. (**C**) The activity of the full-length PLCδ1 (○,●) and the deletion mutant lacking the PH domain (□,■) was analysed in the absence (●,■) or presence (○,□) of PI-TPα at 10 μM Ca^{2+} ions. The concentration of PI-TPα was 100 μg/ml. All experiments are representative of at least two experiments performed and presented as described in the previous figure legends.

cellular membrane, the hydrophobic ridge and the C2 domain. A function of Ca^{2+} ion-dependent phospholipid binding has been suggested for several C2 domains [34]. Among those, the first C2 domain of synaptotagmin I has been well characterized. Binding of Ca^{2+} ions to the specific site within the synaptotagmin C2 domain occurred at Ca^{2+} ion concentrations of 1–10 μM and mediated the formation of a ternary complex with phospholipids [19]. Comparison of the Ca^{2+} ion-binding sites of PLCδ1 and synaptotagmin revealed a striking similarity in the residues involved in the co-ordination of Ca^{2+} ions that could be bound to the C2 domains [15]. In experiments presented here (Figure 4B and 5C) an increase in Ca^{2+} ion concentration (10 μM) necessary to detect any activity of ΔPH-PLCδ1 was not sufficient to restore levels of inositol lipid hydrolysis obtained with the full-length protein. These results suggest that the affinity of Ca^{2+} ion-dependent phospholipid binding by PLCδ1 C2 domain was not sufficiently high to mediate membrane attachment independently in the absence of the PH domain.

Table 2 Inositol lipid hydrolysis by PLCδ1 in the presence of isolated C2 domain from synaptotagmin I

HL-60 cells were permeabilized for 40 min and then analysed for inositol phosphate production in the presence of purified PLCδ1 (10 μg/ml) and increasing concentrations of synaptotagmin I C2 domain (Syt C2) at 10 μM Ca^{2+}. The permeabilized cells were incubated for 20 min at 37 °C. The results are averages of duplicate incubations that did not vary by more than 10%. The results presented here are representative of three experiments.

Addition	Inositol phosphates produced (d.p.m.)
None	4697
PLCδ	16767
PLCδ + 10 μg/ml Syt C2	19118
PLCδ + 50 μg/ml Syt C2	17303
PLCδ + 100 μg/ml Syt C2	17339

An effect of the isolated C2 domain from synaptotagmin on PLCδ1 activity in the presence of 10 μM Ca^{2+} ions was also analysed in permeabilized HL-60 cells. The activity of PLCδ1 in the absence of synaptotagmin C2 domain was not decreased in the presence of up to 60-fold molar excess of this domain (Table 2). However, these experiments cannot exclude a possible interaction between the C2 domain within PLCδ1 with the membrane because the absence of competition could be a consequence of a large membrane-binding surface available to this domain.

To examine the possibility that membrane attachment by the PH domain involves its binding to PtdIns(4,5)P_2 we measured the activity of PLCδ1 in the presence of increasing concentrations of Ins(1,4,5)P_3 (Figure 4C, left panel). A 50% decrease in PLCδ1 activity by Ins(1,4,5)P_3 was achieved within a physiological range of Ins(1,4,5)P_3 concentrations (approx. 1 μM). Inhibition of PLCδ1 within this concentration range is selectively directed towards the PH domain, which has a much higher affinity for PtdIns(4,5)P_2 and Ins(1,4,5)P_3 {K_d [PtdIns(4,5)P_2] $\approx$ 1.7 μM} than the catalytic domain {K_d [PtdIns(4,5)P_2] 0.1 mM}. Inhibition at higher concentrations of Ins(1,4,5)P_3 (100 μM) could involve both the PH domain and the catalytic domain. When PLCδ1 activity was analysed in the presence of the deletion mutant retaining an intact PH domain but lacking a large proportion of the rest of the molecule (PLCδ1 PH domain), 50% inhibition was observed at approx. 20-fold molar excess of the PH domain protein (Figure 4C, right panel). The most likely explanation of this effect is the PH domain's binding to PtdIns(4,5)P_2, resulting in a decrease in the number of PtdIns(4,5)P_2 molecules available for protein anchoring and substrate hydrolysis by PLCδ1.

Stimulation of PLCδ1 activity by PI-TP

To analyse the effect of PI-TP on Ca^{2+} ion-stimulated PLCδ1 activity in HL-60 cells, increasing concentrations of PI-TPα

(1–300 μg/ml) were included in the assay. The highest activity of PLCδ1 was measured at PI-TP concentrations (100 μg/ml) previously used for reconstitution of PLCβ and PLCγ activities [20,22]. However, in HL-60 cells the enhancement of PLC activity (approx. 2-fold) was less pronounced than in PC-12 cells (approx. 10-fold). Stimulation of PLCδ1 activity was observed over the range of increasing Ca^{2+} ion concentrations (Figure 5A) but, unlike the activity of PLCδ1 in the presence of PI-TP in PC-12 cells (Table 1), did not seem to approach a maximum at 1 μM Ca^{2+} ions.

The addition of PI-TP, in the presence of Ins(1,4,5)P_3 or the PH domain, increased the activity of PLCδ1 but did not suppress the inhibitory effect of these molecules; similarly the substrate specificity of PLCδ1 was unaffected by PI-TP (results not shown). The effect of PI-TP on Ca^{2+} ion-stimulated PLCδ1 was analysed in a time-course experiment (Figure 5B). An increase in PLC activity by PI-TP was more pronounced at later times (approx. 2-fold stimulation after 15 and 30 min) than early after Ca^{2+} ion stimulation (approx. 0.2-fold increase after 5 min). Taken together, these results are consistent with the proposed function of PI-TP to supply and favourably present substrate that would otherwise become limiting.

Several mechanisms for this PI-TP function have been suggested. One possibility would be that, by analogy with the proposed action of the GM-2 activator of hexosaminidase, PI-TP presents substrate directly to enzymes that hydrolyse and modify PtdIns(4,5)P_2. In this case, an enzyme interaction with the membrane could be by-passed by PI-TP. To test this possibility, the full-length PLCδ1 and ΔPH-PLCδ1 unable to interact with the plasma membrane [18] were compared (Figure 5C). PI-TP was able to increase PLCδ1 activity even at high concentrations (100 μg/ml) of the full-length protein. When ΔPH-PLCδ1 was used at high protein concentration, stimulation by Ca^{2+} ions could be detected. However, the activity of ΔPH-PLCδ1 was not stimulated further by the addition of PI-TP. These experiments therefore suggest that access to a PtdIns(4,5)P_2 pool enriched by PI-TP requires PH domain-mediated interaction with cellular membranes. A possibility that PLCδ1 PH domain interacts with PtdIns(4,5)P_2 bound to PI-TP is less likely. PI-TP function is not limited to enzymes with the PH domain; among those that have it, ligand-binding properties might not be conserved.

Physiological context of PLCδ1 stimulation by Ca^{2+} ions

The Ca^{2+} ion dependence of PLC activation *in vivo* has been extensively studied in different cell types. In many cellular systems, hormone-induced inositol lipid hydrolysis was observed at low intracellular Ca^{2+} levels of unstimulated cells and was insensitive to the removal of extracellular Ca^{2+} ions [35–38]. However, in many systems such as pancreatic islets, spermatozoa, adrenal chromaffin cell, neuroblastoma cells and cerebral cortical slices, stimulation of PLC activity occurred at higher Ca^{2+} ion concentrations and was dependent on Ca^{2+} influx from extracellular medium [39–44]. Depolarization of the cellular membrane by high concentrations of K^+ [45–47], treatment with Ca^{2+} ionophores [48,49] or changes in Ca^{2+} ion concentrations in preparations of permeabilized cells [39], could also lead to an increase in inositol lipid hydrolysis. It has been suggested that an increase in Ca^{2+} ion levels linked to inositol lipid hydrolysis could be achieved by different mechanisms and could involve the opening of receptor-operated Ca^{2+} channels or voltage-dependent Ca^{2+} channels [24]. Results presented here, demonstrating the selective stimulation of PLC isoenzymes by Ca^{2+} ions (Figure 2), suggest that this Ca^{2+} effect is likely to be mediated by PLCδ rather than by a general stimulatory effect on all PLC isoenzymes. Consequently, observed discrepancies between Ca^{2+} ion concentrations required to activate PLC in different cell types could be due to the expression of different PLC isoenzymes. Although PLCδ1 is clearly present in excitable tissues such as brain [23] and the highest levels are detected in skeletal muscle [50], at present there are insufficient data to allow a comparison between levels of PLCδ1 expression (or other PLCδ isoenzymes) and the ability of different cells to hydrolyse inositol lipids at higher Ca^{2+} ion concentrations. Nevertheless the results obtained from the analysis of CHO cells stably transfected with PLCδ1 are consistent with the suggested function for this isoenzyme [12]. In this study the cells were treated with thrombin, an agonist known to elevate intracellular Ca^{2+} ion levels by stimulating Ca^{2+} influx. In transfected CHO cells, thrombin-stimulated inositol lipid hydrolysis was most pronounced at later times (30 min after stimulation) and the stimulation was completely abolished by the removal of Ca^{2+} ions from the extracellular medium. Furthermore the treatment of cells with Ca^{2+} ionophore alone caused a substantial increase in PLC activity in transfected cells with very little effect on control CHO cells.

It has been previously speculated [12] that in some systems the stimulation of PLCδ1 by Ca^{2+} ions could be secondary to the stimulation of PLCβ or PLCγ isoenzymes, leading to prolonged hydrolysis of inositol lipids. When discussing this possibility it has to be considered that activation of PLC isoenzymes results not only in an increase in Ca^{2+} ion concentrations in the cytoplasm but also changes cellular concentrations of Ins(1,4,5)P_3 and PtdIns(4,5)P_2. Experimental evidence has now accumulated (Figure 4) [13,14,32] to suggest that an increase in Ins(1,4,5)P_3 concentrations, accompanied by a decrease in the number of PtdIns(4,5)P_2 molecules available to the enzyme, could limit the association of PLCδ1 with cellular membrane and its subsequent activation. Therefore a possible relationship between the activation of PLCβ or PLCγ isoenzymes and the activation of PLCδ1 could be more complex than previously envisaged.

Studies of PLCδ1 also suggested that the stimulation by Ca^{2+} ions, sufficient to activate the enzyme, could be further potentiated or modified through interaction with other protein components [10–12]. In preparations of permeabilized CHO cells expressing PLCδ1, GTP[S] further increased inositol lipid hydrolysis, suggesting the involvement of a G-protein [12]. The nature of this G-protein and whether it interacts directly with PLCδ1 have not been clarified. In another recent study [11] a PLC enzyme present in complexes with the novel type of G-protein, G_h, was identified as a proteolytic fragment of PLCδ1. The α subunit of this heterodimeric G-protein is characterized by transglutaminase activity in addition to its GTP-binding function. The regulation of PLCδ1 by $G_h\alpha$ seems to be different from the regulation of PLCβ isoenzymes by the subunits of heterotrimeric G-proteins when analysed in a similar system *in vitro*. The addition of $G_h\alpha$ did not change the maximum of substrate hydrolysis but the maximum was reached at a somewhat lower Ca^{2+} ion concentration (10 μM instead of 20 μM) [51]. A physiological significance of this observation *in vitro* and the possible coupling of PLCδ1 with receptors that interact with G_h proteins (e.g. α_{1B} adrenoceptors) clearly require further studies.

A physiological meaning of the requirement for high Ca^{2+} ion concentrations to produce the Ins(1,4,5)P_3 molecule with the main biological role of mediating an increase in Ca^{2+} ion levels has been previously questioned and referred to as the 'calcium paradox' [4]. Because the hydrolysis of inositol lipids results in the production of DG as another second messenger, a preference of a PLC for phosphatidylinositol would result in the production of DG independently of Ins(1,4,5)P_3. Our experiments demon-

strated that PLCδ1 could preferentially hydrolyse polyphosphoinositides to produce Ins(1,4,5)P_3 (Figure 3). However, this substrate preference, at least in evolutionary terms, might not be linked to Ins(1,4,5)P_3 production and Ca^{2+} mobilization. In lower eukaryotes, where PLCδ-like molecules seem to be the only PLC isoenzyme, there is little evidence for Ins(1,4,5)P_3 mediated functions; to our knowledge, there is no report of an Ins(1,4,5)P_3 receptor. The function of DG produced by PtdIns(4,5)P_2 hydrolysis in lower eukaryotes also remains unclear. For example, the PKC enzyme in *Saccharomyces cerevisiae* is different from the main mammalian isoenzymes in a requirement for Rho GTPase for its activation and, in this case, the function of DG could be only modulatory [52]. One role for PtdIns(4,5)P_2 that seems to be conserved between lower and higher eukaryotes is related to the binding of proteins important for organization of the cytoskeleton, and to regulation of their function by changes in concentration of PtdIns(4,5)P_2 [2,53]. PtdIns(4,5)P_2 levels are, at least in part, determined by PtdIns(4,5)P_2 hydrolysis by specific PLCs and this could be an important PLC function that occurred early in the course of evolution. In mammalian cells, where the complexities of Ca^{2+} ion functions and the consequences of PtdIns(4,5)P_2 hydrolysis are becoming apparent, a Ca^{2+}-regulated PLC could be integrated in the control of cellular functions in many different ways.

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