Ceramide dissociates 3′-phosphoinositide production from pleckstrin homology domain translocation

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Numerous hormones, cytokines and transforming oncogenes activate phosphoinositide 3-kinase (PI-3K), a lipid kinase that initiates signal transduction cascades regulating cellular proliferation, survival, protein synthesis and glucose metabolism. PI-3K catalyses the production of the 3′-phosphoinositides PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, which recruit downstream effector enzymes to the membrane via their pleckstrin homology (PH) domains. Recent studies have indicated that another signalling lipid, the sphingolipid ceramide, inhibits several PI-3K-dependent events, including insulin-stimulated glucose uptake and growth-factor-stimulated cell survival. Here we show that ceramide analogues specifically prevent the recruitment of the PtdIns(3,4,5)P_3-binding proteins Akt/protein kinase B (PKB) or the general receptor for phosphoinositides-1 (GRP1). Specifically, the short-chain ceramide derivative C2-ceramide inhibited the platelet-derived growth factor (PDGF)-stimulated translocation of full-length Akt/PKB, as well as truncated proteins encoding only the PH domains of Akt/PKB or GRP1. C2-ceramide did not alter the membrane localization of the PH domain for phospholipase Cδ, which preferentially binds PtdIns(4,5)P_2, nor did it affect the PDGF-stimulated production of PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3. Interestingly, a glucosylceramide synthase inhibitor, 1-phenyl-2-decanoylamino-3-morpholinopropan-1-ol (PDMP), shown previously to increase intracellular ceramide concentrations without affecting PI-3K [Rani, Abe, Chang, Rosenzweig, Saltiel, Radin and Shayman (1995) J. Biol. Chem. **270**, 2859–2867], recapitulated the inhibitory effects of C2-ceramide on PDGF-stimulated Akt/PKB phosphorylation. These studies indicate that ceramide prevents the translocation of certain PtdIns(3,4,5)P_3-binding proteins, despite the presence of a full complement of PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3. Furthermore, these findings suggest a mechanism by which stimuli that induce ceramide synthesis could negate the fundamental signalling pathways initiated by PI-3K.

Key words: Akt, GLUT4 translocation, phosphoinositide 3-kinase, protein kinase B, sphingolipids.

INTRODUCTION

Phosphoinositide 3-kinase (PI-3K) is an obligate intermediate transducing the metabolic [1], anti-apoptotic [2] and mitogenic [3] effects of insulin and other growth factors. PI-3K phosphorylates specific phosphoinositides to generate the lipids PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. These phosphorylated products recruit downstream effector enzymes such as Akt/protein kinase B (PKB), the general receptor for phosphoinositides-1 (GRP1) and phosphoinositide-dependent kinase 1 (PDK1) to the plasma membrane [4]. GRP1 catalyses guanine nucleotide exchange on ADP-ribosylation factors (Arfs) [5,6], which are small GTPases implicated in vesicular trafficking. PDK1 is a serine/threonine kinase that phosphorylates downstream kinases such as Akt/PKB and pp70 S6-kinase on regulatory residues in their catalytic domains [7,8]. Akt/PKB is also a serine/threonine kinase that regulates numerous proteins, including transcription factors, anti-apoptotic proteins and metabolic enzymes [9]. The pleckstrin homology (PH) domains in these proteins bind tightly to PtdIns(3,4,5)P_3 [10], producing active signalling complexes at the plasma membrane. For example, PDK1 and Akt/PKB translocate to the plasma membrane via binding to PtdIns(3,4,5)P_3, which facilitates the phosphorylation of Akt/PKB by the constitutively active PDK1 [11].

The sphingomyelin derivative ceramide is a signalling molecule implicated in a broad array of cellular events relating to growth and differentiation [12–14]. Most studies on ceramide have evaluated its possible role in the initiation of apoptosis (reviewed in [14]). Numerous apoptotic stimuli (e.g. cytokines, cytotoxic agents and environmental stresses) reportedly increase intracellular ceramide levels; the addition of ceramide analogues to isolated cells triggers apoptosis [14]. However, the interpretation of these results has been controversial [15,16]. Nevertheless, several additional studies suggest that ceramide is required for the induction of apoptosis by at least certain stimuli. First, studies with sphingomyelinase knock-out mice confirm that ceramide accumulation is required for optimal endothelial cell apoptosis in response to ionizing radiation [17]. Secondly, the prevention of ceramide synthesis with the compound fumonisin B identifies a role for the sphingolipid in fatty-acid-induced β-cell apoptosis [18] or daunorubicin-induced leukaemia cell apoptosis [13]. Thirdly, overexpression of glucosylceramide synthase, which lowers cellular ceramide levels by catalysing its glucosylation,

prevents apoptosis induced by the chemotherapeutic agent adriamycin [19]. Studies with ceramide analogues also implicate the sphingolipid in various other biological processes: (1) ceramide analogues stimulate differentiation of several cell types [13], (2) ceramide analogues regulate growth arrest after serum withdrawal [20,21], and (3) ceramide analogues inhibit insulin-stimulated glucose metabolism [22,23].

As is apparent from the above discussions, the biological activities of these two classes of messenger (i.e. ceramide and 3′-phosphoinositides) are often in direct opposition. For example, in numerous cell types PI-3K supports survival [2], whereas ceramide is implicated in apoptosis [14]. Similarly, in insulin-responsive cells PI-3K stimulates glucose uptake [1], whereas ceramide opposes it [22–24]. These observations prompted the hypothesis that ceramide inhibits PI-3K signalling; studies have confirmed that it antagonizes the PI-3K-dependent phosphoryl-ation and activation of Akt/PKB [22,25–27]. However, in those studies ceramide did not inhibit PI-3K activity; its effect on Akt/PKB is unlikely to explain all of its effects against PI-3K. This is particularly true in the studies on glucose uptake, because the expression of dominant-negative Akt/PKB constructs results in an even greater inhibition of Akt/PKB than can be achieved with ceramide but has no effect on glucose metabolism [28]. Here we describe a partial mechanism for the ceramide effect not only on Akt/PKB phosphorylation but also on other signalling events downstream of PI-3K. Ceramide analogues prevented the membrane localization of the PH domains for Akt/PKB and GRP1, which bind PtdIns$(3,4,5)P_3$, but not the PH domain for phospho-lipase Cδ (PLCδ), which binds PtdIns$(4,5)P_2$. These ceramide analogues did not alter the accumulation of 3′-phosphoinositides, indicating that this inhibition occurs via a previously undefined mechanism unrelated to 3′-phosphoinositide production. Collectively, these studies suggest a mechanism by which ceramide analogues could oppose the growth-promoting and metabolic actions of PI-3K.

EXPERIMENTAL

Antibodies and reagents

Polyclonal antibodies directed against the phosphorylated forms of mitogen-activated protein kinase (MAPK) and Akt/PKB (S473 site) were from Promega (Madison, WI, U.S.A.) and Cell Signaling Technology (Beverly, MA, U.S.A.) respectively. Antibodies recognizing phosphatase and tensin homologue (PTEN) were from Oncogene (Cambridge, MA, U.S.A.); antibodies against haemagglutinin (HA) were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Secondary anti-rabbit antibodies coupled to Texas Red or horseradish peroxidase were from Jackson Immunoresearch Laboratories (West Grove, PA, U.S.A.) or Santa Cruz Biotechnology respectively.

C2-ceramide, C2-dihydroceramide and D,L-*threo*-1-phenyl-2-decanoylamino-3-morpholinopropan-1-ol (PDMP) were from Calbiochem-Novabiochem (La Jolla, CA, U.S.A.). Pig insulin, human and rat PDGF, and nystatin were from Sigma (St Louis, MO, U.S.A.). *myo*-[2-³H]Inositol was from Amersham-Pharmacia Biotech (San Francisco, CA, U.S.A.).

Cell culture

NIH-3T3 or 3T3-L1 fibroblasts were maintained in DMEM (Dulbecco's modified Eagle's H21 medium) supplemented with 10 % (v/v) calf serum. 3T3-L1 fibroblasts expressing HA–Akt (β-isoform) and empty vector were periodically selected in 800 μg/ml G418; they were as described previously [29]. PC3 cells lacking PTEN were obtained from Richard Roth (Stanford,

CA, U.S.A.) and are described elsewhere [30]. These cells were maintained in RPMI-1640 medium containing 10 % (v/v) fetal bovine serum containing antibody mix (penicillin/streptomycin/fungizone) from Biowhittaker (Walkersville, MD, U.S.A.).

cDNA constructs

Mark Lemmon (University of Pennsylvania, Philadelphia, PA, U.S.A.) provided constructs encoding the PH domain of GRP1 coupled to green fluorescent protein (GFP) [10], Sandra Watton and Doreen Cantrell (Imperial Cancer Research Fund, London, U.K.) provided the PH domain of Akt/PKB coupled to GFP [31], and Tamas Balla provided the PH domain of PLCδ coupled to GFP [32].

Analysis of localization of GFP–GRP-PH, GFP–Akt-PH and GFP–PLCδ-PH in NIH-3T3 fibroblasts

NIH-3T3 fibroblasts were grown to 20–30 % confluence on coverslips. Cells were always fed 2 h before transfection. Cells were transfected with 15 μg of cDNA encoding GFP–GRP-PH, GFP–Akt-PH or GFP–PLCδ-PH by precipitation with calcium phosphate as described in Current Protocols in Molecular Biology (2000) on CD-ROM (John Wiley and Sons, New York). Cells were analysed by fluorescence microscopy 20–24 h after transfection. Digital image processing was performed as described previously with Metamorph software [22].

Measurement of PtdIns$(3,4)P_2$ and PtdIns$(3,4,5)P_3$ by HPLC

NIH-3T3 fibroblasts were grown to at least 60 % confluence in 75 cm² flasks. Cells were washed and labelled for 36 h with *myo*-[2-³H]inositol (20 μCi/ml in inositol-free DMEM) containing 10 % (v/v) calf serum. After 24 h the medium was removed and replaced with fresh *myo*-[2-³H]inositol medium. Cells were serum-deprived for 2 h in inositol-free and serum-free DMEM containing 0.2 % BSA and 10 μCi/ml *myo*-[2-³H]inositol. Cells were then treated with PDGF, C2-ceramide or C2-dihydroceramide as indicated in the Figure legends. Extraction and deacylation of lipids were performed as described previously [33]. The glycero-phosphoinositol phosphates were analysed by HPLC as described [33].

Immunofluorescence

After the treatment indicated in the Figure legends, 3T3-L1 fibroblasts expressing HA–Akt (β isoform) were fixed in 3 % (w/v) paraformaldehyde and washed as described previously [34]. Immunofluorescence on permeabilized cells was performed as described [34]. Images were captured with a digital camera and Metamorph imaging software.

Immunoblot analyses of total cell lysates

Cells grown to confluence in 100 mm diameter dishes were serum-deprived as indicated in the Figure legends. Cells were washed twice with ice-cold PBS and lysed in 100 μl of 66 mM Tris/HCl, pH 8.0, containing 2 % (w/v) SDS and 100 mM vanadate. Samples were boiled for 2 min and DNA was sheared by passing extracts several times through a 27-gauge needle. Insoluble material was pelleted by centrifuging the samples for 30 min at 20 000 g. Protein concentrations were determined with the bicinchoninic acid protein assay kit from Pierce; 40 μg of total protein was loaded into each well of a 7.5 % or 10 % (w/v) PAGE gel. Proteins were transferred to nitrocellulose and probed with the indicated antibodies as described [35]. Antibody

detection was performed with the enhanced chemiluminescence kit from Amersham.

RESULTS

The effects of ceramide on PDGF signalling were investigated with a short-chain ceramide analogue, C2-ceramide. C2-ceramide disperses easily in culture medium while retaining the capacity to traverse the cell membrane; this compound has been used by numerous laboratories to identify potential roles for ceramide in a broad array of cellular events. We have previously demonstrated that C2-ceramide selectively inhibits Akt/PKB phosphorylation without affecting PI-3K activity [22]; Figures 1(A) and 1(B) illustrate the dose response and time course for the effect. C2-ceramide inhibits Akt/PKB phosphorylation within 10 min of its addition to NIH-3T3 fibroblasts, with maximal inhibition being obtained at a dose of 100 μM. Experiments were also conducted with an inhibitor of glucosylceramide synthase to induce endogenous ceramide accumulation by preventing its glucosylation [36]. This compound, termed PDMP, stimulates ceramide accumulation (approx. 4-fold after 24 h) and cell cycle

A

α-PAkt

| 0 | 0 | 10 | 25 | 50 | 100 | C2 (μM) |
| - | + | + | + | + | + | PDGF |

B

α-PAkt

| 0 | 0 | 5 | 10 | 15 | 30 | C2 (Time in min.) |
| - | + | + | + | + | + | PDGF |

C

α-PAkt

α-PMAPK

| - | - | + | + | PDMP |
| - | + | - | + | PDGF |

D

α-PAkt

| 0 | 0 | 25 | 25 | 100 | 100 | C1P (μM) |
| - | + | - | + | - | + | PDGF |

Figure 1 Ceramide and PDMP inhibit Akt/PKB phosphorylation

(**A**) NIH-3T3 fibroblasts were serum-starved in DMEM containing 0.2% BSA for 2 h before treatment with the indicated dose of C2-ceramide (C2) for 20 min. After a 10 min stimulation with (+) or without (−) (100 ng/ml) PDGF, cells were lysed and proteins were resolved by SDS/PAGE and immunoblotted with antibodies recognizing the phosphorylated Ser-473 residue of Akt (αP-Akt). (**B**) Cells were treated as in (**A**) above except that they were incubated for various durations (as indicated in the Figure) with a maximal dose (100 μM) of C2-ceramide. (**C**) Cells were treated as in (**A**) above except that they were pretreated for 48 h in DMEM containing 1% (v/v) calf serum with (+) or without (−) 50 μM PDMP. To confirm the specificity of the drug, samples were also immunoblotted with antibodies recognizing the phosphorylated p42 and p44 forms of MAPK (αPMAPK). (**D**) Cells were treated as in (**A**) above except that selected samples were incubated with the indicated dose of C8-ceramide 1-phosphate (C1P). Results in each panel are representative of at least two independent experiments.

arrest in NIH-3T3-IR fibroblasts but does not affect either MAPK or PI-3K activity [36]. As described in Figure 1(C), PDMP specifically inhibited PDGF-stimulated Akt/PKB phosphorylation in NIH-3T3 fibroblasts, recapitulating the effect observed with exogenous ceramide analogues (Figure 1C, upper panel). As expected, PDMP had no inhibitory effect on PDGF-stimulated MAPK phosphorylation (Figure 1C, lower panel).

A concern when using exogenous ceramide analogues is that they can be converted into other ceramide metabolites that are actually the relevant signalling molecules mediating a particular biological effect [14]. One such metabolite is ceramide 1-phosphate (C1P), which is produced in response to various stimuli by the enzyme ceramide kinase [37]. Using a short-chain C1P analogue, C8-ceramide 1-phosphate, we tested whether this phosphorylated product mimicked the C2-ceramide effect on PDGF-stimulated Akt/PKB phosphorylation. C8-ceramide 1-phosphate, under conditions shown to increase cellular C1P concentrations [38], had no effect on PDGF-stimulated Akt/PKB phosphorylation (Figure 1D). We also tested a much higher (4-fold) dose but again saw no effect (Figure 1D). Collectively, the studies shown in Figure 1 suggest that a physiologically relevant increase in the intracellular concentration of ceramide, but not C1P, selectively blocks Akt/PKB phosphorylation.

PDGF stimulates Akt/PKB phosphorylation by promoting its translocation, as well as that that of one or more up-stream kinases, to plasma membrane domains enriched in PtdIns(3,4,5)P_3 [4,11]. To determine whether C2-ceramide blocked the PDGF-stimulated recruitment of Akt/PKB to the plasma membrane, we used a stable 3T3-L1 fibroblast cell line overexpressing a HA-tagged Akt/PKB derivative (HA–Akt). Previous studies with these fibroblasts had indicated that both insulin and PDGF stimulate HA–Akt phosphorylation and activation [29]. Our results demonstrate that stimulation of these cells with PDGF caused a marked translocation of HA–Akt/PKB from the cytoplasm to the plasma membrane. C2-ceramide, under conditions identical with those required for the inhibition of Akt/PKB phosphorylation, prevented this redistribution of HA–Akt/PKB (Figure 2).

Akt/PKB interacts with phosphoinositides via its PH domain, a 106-residue region with a high affinity for PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 [39]. We next sought to determine whether ceramide also blocked the translocation of a truncated PH-domain construct. Experiments were therefore conducted with the PH domain from Akt/PKB tagged with GFP (GFP–Akt-PH) [40]. After transfection of the GFP–Akt-PH construct into NIH-3T3 fibroblasts, the cells were treated with or without PDGF or C2-ceramide, fixed in paraformaldehyde and detected by fluorescence microscopy. C2-ceramide blocked the trans-location of this PH-domain construct (Figure 3).

Questions persist regarding whether the ceramide effect on PI-3K-dependent signalling is solely the result of this inhibition of Akt/PKB or is a more general effect on multiple PtdIns(3,4,5)P_3-binding proteins. We therefore sought to determine whether the C2-ceramide effect applied more generally to the PH domains of other PtdIns(3,4,5)P_3-binding proteins and specifically evaluated whether C2-ceramide altered the PDGF-induced translocation of the PH domain of GRP1. In binding assays, the GRP1-PH domain has a high affinity and specificity for PtdIns(3,4,5)P_3 but not for PtdIns(3,4)P_2 [10]. This is slightly different from the PH domain of Akt/PKB, which has equal affinity for either phospho-lipid. When cDNA encoding a GFP-tagged PH domain of GRP1 (GFP–GRP-PH) [10] was introduced into NIH-3T3 fibroblasts by transfection, most GFP–GRP-PH localized to the nucleus [10,41]. Stimulation with PDGF induced the translocation of GFP–GRP-PH to the plasma membrane [10,41,42]. Pretreating
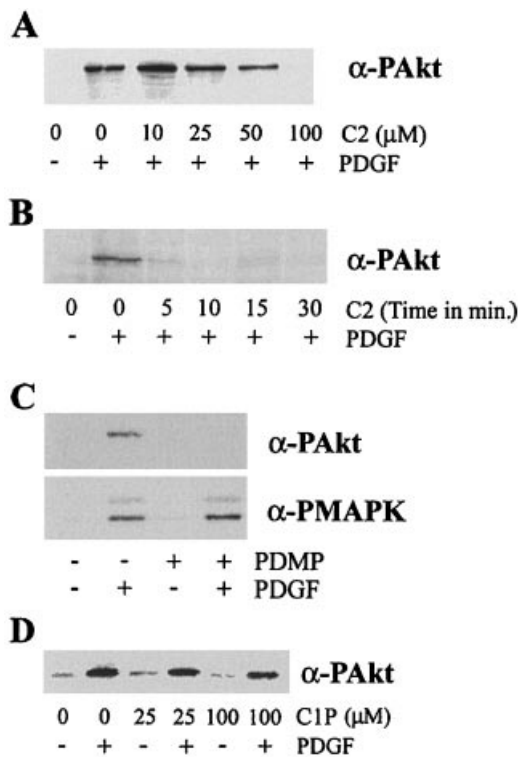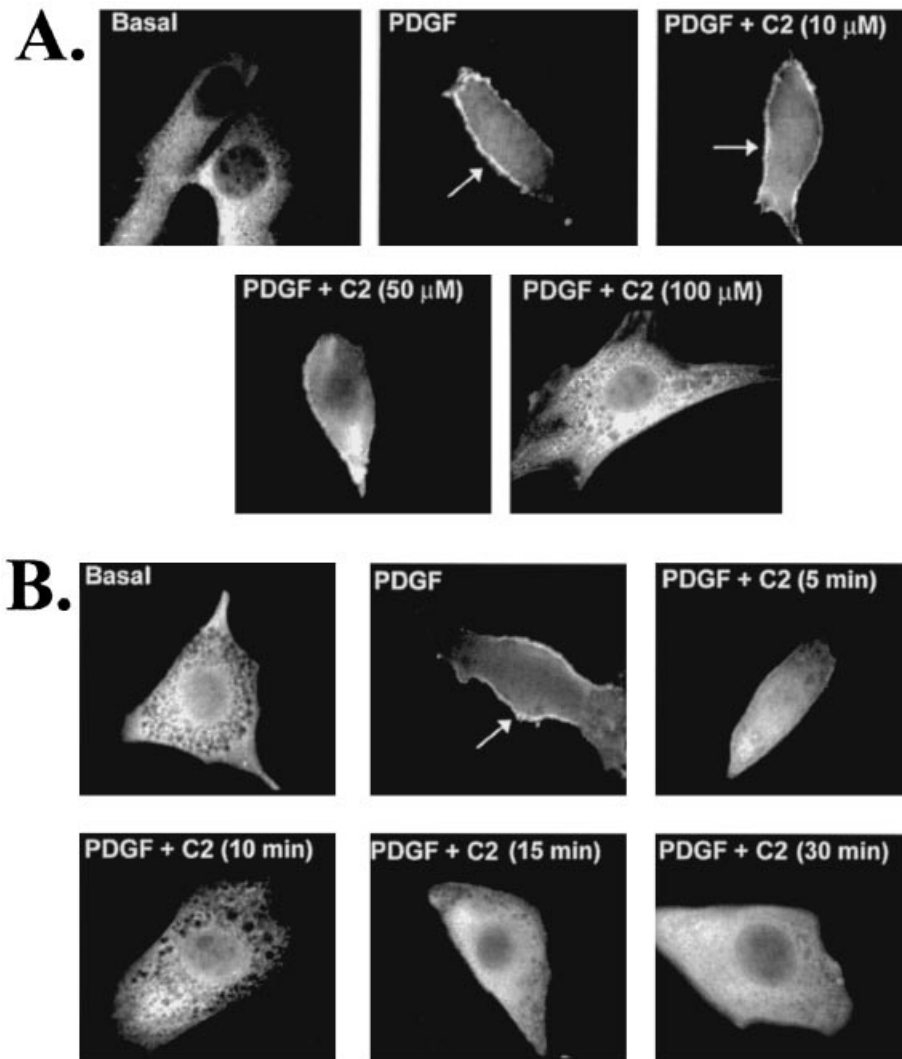
Figure 2   Ceramide inhibits Akt/PKB translocation

To facilitate the removal of Akt/PKB from the plasma membrane, 3T3-L1 fibroblasts expressing HA-tagged Akt/PKB were serum-deprived for 2 h in DMEM containing 0.2% BSA and 100 μM LY294002. LY294002 was removed by washing in PBS; the cells were kept in DMEM containing 0.2% BSA for subsequent treatments. (**A**) Cells were treated with or without the indicated dose of C2-ceramide (C2, 20 min) before stimulation with or without PDGF for 5 min. HA–Akt/PKB was detected by immunofluorescence with anti-HA antibodies (αHA) as described in the Experimental section. Arrows indicate membrane-localized full-length Akt/PKB. Results are representative of five independent experiments. (**B**) Cells were treated as in (**A**) except that C2-ceramide (100 μM) was added for the indicated duration before the addition of PDGF.
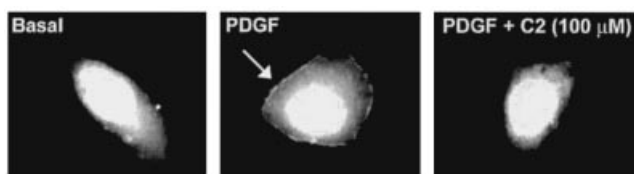

Figure 3   Ceramide inhibits GFP–Akt-PH translocation

NIH-3T3 fibroblasts transfected with GFP–Akt-PH were serum-deprived in DMEM containing 0.2% BSA for 2 h before treatment with C2-ceramide (C2, 100 μM) 20 min before the addition of PDGF (5 min). GFP–Akt-PH was detected by fluorescence microscopy. Arrows indicate membrane-localized GFP–Akt-PH. Results are representative of two independent experiments.

cells with the PI-3K inhibitor wortmannin or C2-ceramide, but not the biologically inactive C2-dihydroceramide [43], inhibited GFP–GRP-PH translocation (Figure 4). The concentration dependence of this C2-ceramide effect was identical with its effect on Akt/PKB translocation and phosphorylation (Figures 1, 2 and 5A), as was the rate at which the inhibition occurred (Figures 1 and 5B). Moreover, the C2-ceramide effect was observed 30, 60, 90, 180 and 300 s after PDGF stimulation (Figure 6), indicating that C2-ceramide does not merely alter the temporal profile of GFP–GRP-PH translocation.

Interestingly, the C2-ceramide effect seemed reserved for PH domains with an affinity for PtdIns(3,4,5)P_3. The PH domain of PLCδ has been shown to localize to the plasma membrane owing to its high affinity for PtdIns(4,5)P_2 [32]. Unlike the PH domains

Figure 4   C2-ceramide inhibits GFP–GRP-PH translocation

NIH-3T3 fibroblasts transfected with GFP–GRP-PH were serum-starved in DMEM containing 0.2% BSA for 2 h before treatment with or without wortmannin (WT, 100 nM, 15 min), C2-ceramide (C2, 100 μM, 20 min) or C2-dihydroceramide (D-C2, 100 μM, 20 min). Cells were stimulated with PDGF for 2 min and fixed in paraformaldehyde. GFP–GRP-PH was detected by fluorescence microscopy. Arrows indicate membrane-localized GFP–GRP-PH. Results are representative of six independent experiments.
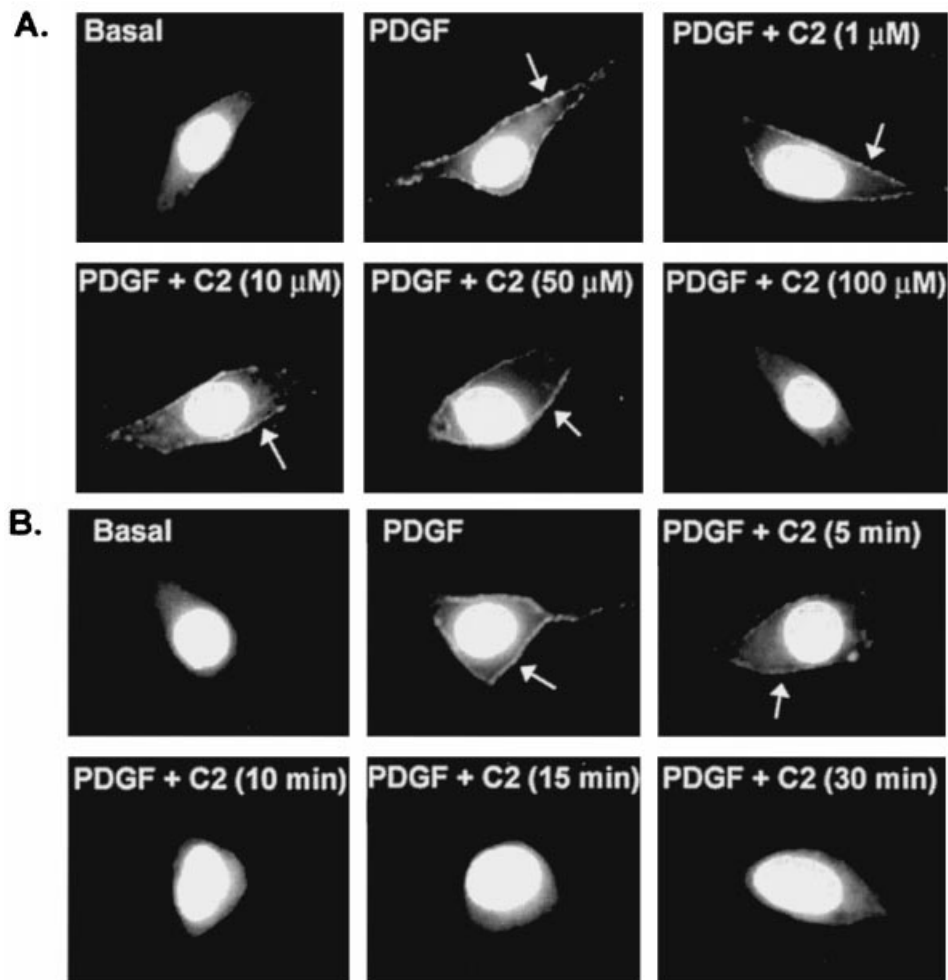

Figure 5   C2-ceramide inhibits GFP–GRP-PH translocation: (A) dose response; (B) time course

(**A**) NIH-3T3 fibroblasts transfected with GFP–GRP-PH were serum-starved in DMEM containing 0.2% BSA for 1 h before treatment with or without the indicated dose of C2-ceramide (C2) for 20 min. Cells were stimulated with PDGF for 2 min and fixed; GFP–GRP-PH was detected by fluorescence microscopy. Arrows indicate membrane-localized GFP–GRP-PH. Results are representative of three independent experiments. (**B**) NIH-3T3 fibroblasts transfected with GFP–GRP-PH were treated as in (**A**) except that they were treated with or without C2-ceramide (C2, 100 μM) for the indicated durations. Results are representative of two independent experiments.

of GRP1 and Akt/PKB, the PH domain of PLCδ associates with the membrane in unstimulated cells, owing to the constitutive presence of PtdIns(4,5)P_2 at the plasma membrane [32]. After the introduction of a GFP-tagged PLCδ fusion protein (GFP–PLC-PH) into NIH-3T3 cells, the protein localized to the cell periphery (Figure 7). Neither C2-ceramide nor wortmannin, at doses that completely blocked GFP–Akt-PH or GFP–GRP-PH translocation, reversed the membrane localization of GFP–PLC-PH (Figure 7).

Although C2-ceramide does not inhibit insulin or growth factor stimulated PI-3K activity [22,23,25,26], we considered the possibility that C2-ceramide could alter 3′-phosphoinositide
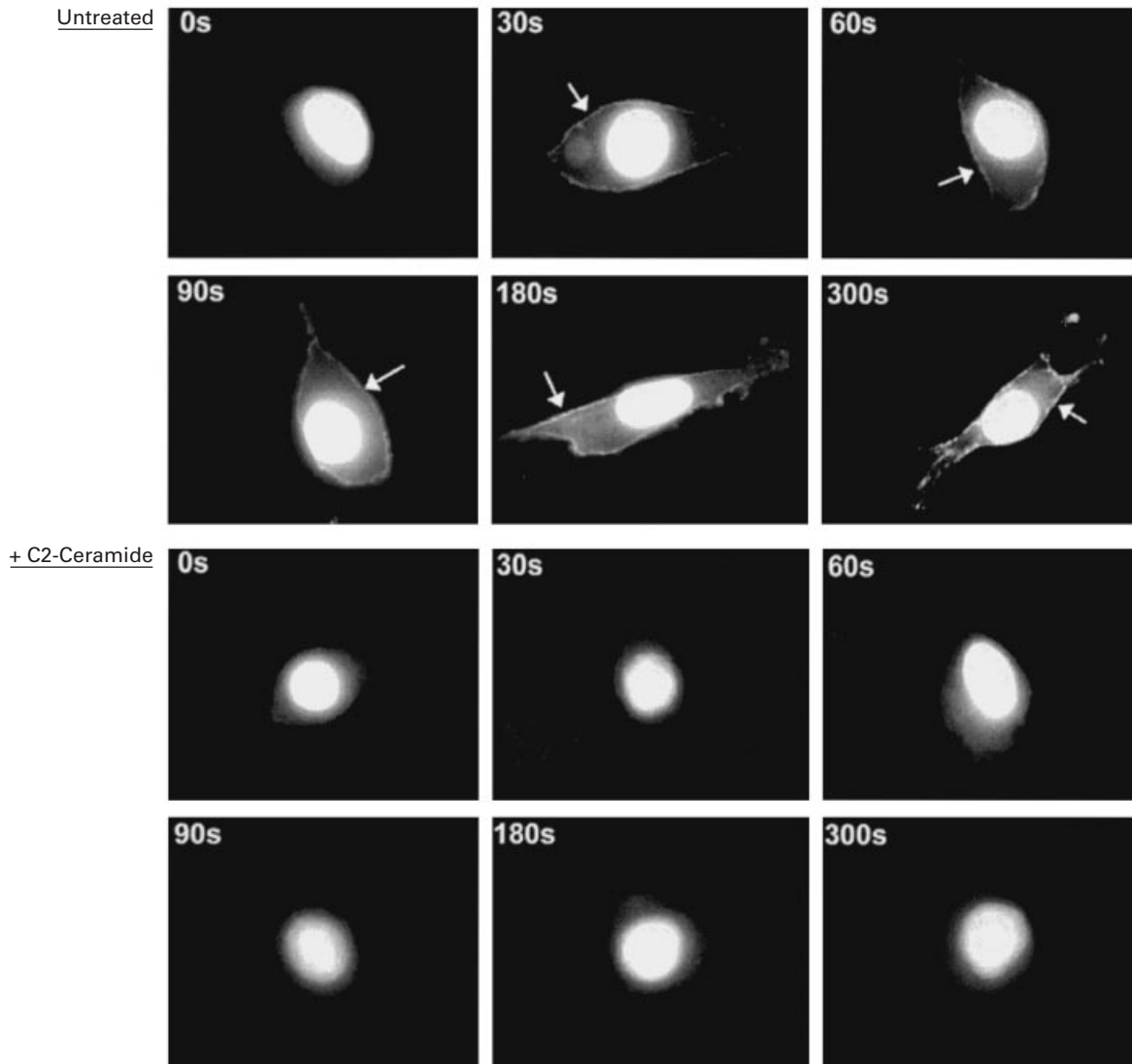
Figure 6   C2-ceramide inhibits GFP–GRP-PH translocation (PDGF-time course)

NIH-3T3 fibroblasts transfected with GFP–GRP-PH were serum-starved in DMEM containing 0.2 % BSA for 2 h before treatment without (untreated) or with (+ C2-ceramide) C2-ceramide (100 μM) for 20 min. Cells were stimulated with PDGF for various durations and fixed in paraformaldehyde. GFP–GRP-PH was detected by fluorescence microscopy. Results are representative of two independent experiments.
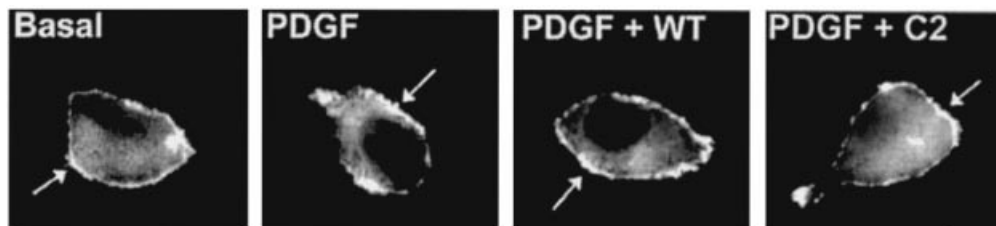

Figure 7   C2-ceramide has no effect on GFP–PLC-PH localization

NIH-3T3 fibroblasts transfected with GFP–PLC-PH were serum-starved in DMEM containing 0.2 % BSA for 2 h before treatment with or without wortmannin (WT, 100 nM) or C2-ceramide (C2, 100 μM) for 20 minutes. Cells were stimulated with PDGF for various durations and fixed; GFP–PLC-PH was detected by fluorescence microscopy. Arrows indicate membrane-localized GFP–PLC-PH. Results are representative of two independent experiments.
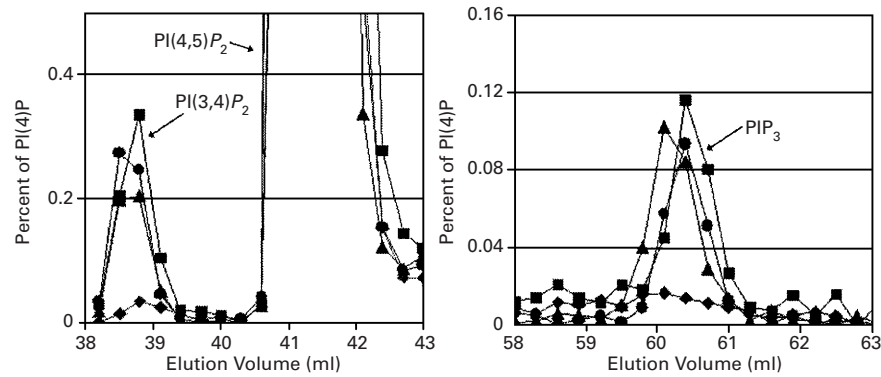
Figure 8 **C2-ceramide has no effect on the accumulation of PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3**

NIH-3T3 fibroblasts labelled with [^{3}H]inositol were serum-deprived in DMEM containing 0.2% BSA for 2 h before treatment with or without C2-ceramide (100 μM) or C2-dihydroceramide (100 μM) for 20 min. After stimulation with PDGF, lipids were extracted and levels of PtdIns(3,4)P_2 (left panel) or PtdIns(3,4,5)P_3 (right panel) were measured as described in the Experimental section. Counts in each fraction were expressed as a percentage of the total counts in the PtdIns4P[PI(4)P] peak, which remained unchanged by the indicated treatments. Results are representative of two independent experiments. Symbols: ◆, basal; ■, PDGF; ▲, PDGF + C2-ceramide; ●, PDGF + C2-dihydroceramide. Abbreviations: PI(4,5)P_2, PtdIns(4,5)P_2; PI(3,4)P$_2$, PtdIns(3,4)P_2; PIP$_3$, PtdIns(3,4,5)P_3.
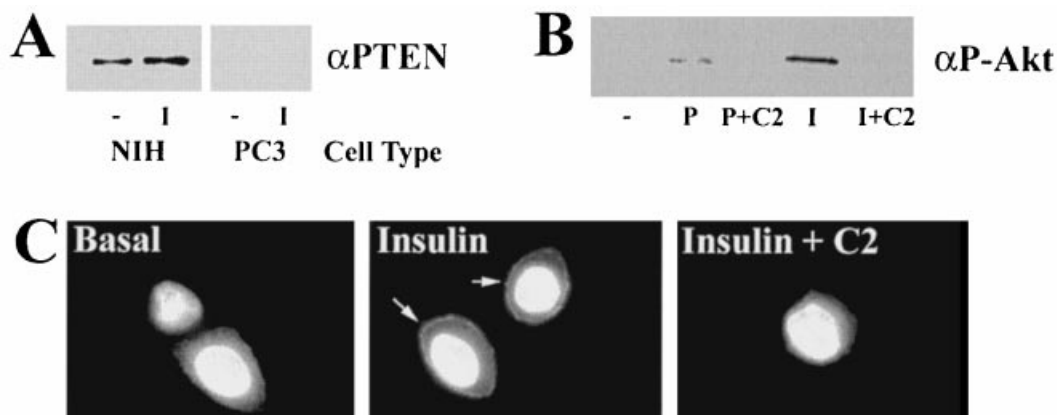

Figure 9 **C2-ceramide effects are not mediated by PTEN**

(**A,B**) PC3 cells were serum starved in DMEM containing 0.2% BSA and LY294002 (200 μM) for 2 h, washed with PBS and placed in DMEM containing 0.2% BSA with (C2) or without (−) C2-ceramide (100 μM) for 20 min. Insulin (I, 1 μM) or PDGF (P, 100 ng/ml) was added for 10 min; cell lysates were prepared as in Figure 1(A). Proteins were detected with antibodies recognizing PTEN (αPTEN) or the phosphorylated (Ser-473) form of Akt (αP-Akt). (**C**) PC3 cells were serum-starved in DMEM containing 0.2% BSA and LY294002 (100 μM) for 2 h, washed with PBS and placed back in DMEM containing 0.2% BSA with (C2) or without C2-ceramide (100 μM, 20 min). Insulin (1 μM) was added for 3 min and cells were fixed. Transfected cells were detected by fluorescence microscopy. Arrows indicate membrane-localized GFP–GRP-PH. Results are representative of two independent experiments.

production by another mechanism (e.g. the activation of a lipid phosphatase such as PTEN [30]). To measure cellular PtdIns(3,4,5)P_3 levels, a well-established method for quantifying PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 *in vivo* was used [33]. In brief, NIH-3T3 fibroblasts were labelled with [^{3}H]inositol before treatment with ceramide, dihydroceramide and PDGF. Subsequently the lipids were extracted, deacylated and resolved by HPLC. Fractions were collected and the [^{3}H]inositol present in each was determined by scintillation counting. As expected, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 levels remained low in unstimulated cells but rose markedly within 3 min of stimulation with PDGF. Neither C2-ceramide nor C2-dihydroceramide had any effect on PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 production (Figure 8). We also tested whether C2-ceramide could affect GFP–GRP-PH translocation or Akt/PKB phosphorylation in cells lacking PTEN. This would indicate whether C2-ceramide might stimulate PTEN to dephosphorylate either general or

localized PtdIns(3,4,5)P_3 microdomains. The absence of PTEN from the prostate cancer cell line PC3 [30] was confirmed by immunoblotting (Figure 9A). To remove residual PI-3K activity before analysis, cells were incubated with the reversible PI-3K inhibitor LY294002, which was removed by washing before treatment with growth factors. Both insulin and PDGF stimulated Akt/PKB phosphorylation (Figure 9B) and GFP–GRP-PH translocation (PDGF results not shown) but insulin was a much better agonist. C2-ceramide blocked the stimulation of both Akt/PKB phosphorylation (Figure 9B) and GFP–GRP-PH translocation (Figure 9C) in these cells, confirming that the effect was independent of PTEN.

DISCUSSION

Short-chain ceramide analogues have been shown to inhibit insulin-stimulated and growth-factor-stimulated Akt/PKB

phosphorylation and activation, as well as other PI-3K-mediated effects [22,23,25–27]. The studies described here help to define the cellular mechanisms governing these ceramide effects. Short-chain ceramide analogues prevented PDGF-induced translocation to the plasma membrane of full-length Akt/PKB, as well as the truncated PH domains of either Akt/PKB or GRP1 (Figures 2–6). The dose response and time course for these effects were identical with those required to prevent Akt/PKB phosphorylation (Figures 1, 2 and 5). In contrast, C2 ceramide did not affect the membrane localization of the PH domain of PLCδ, which preferentially binds PtdIns(4,5)P_2 (Figure 7). Ceramide effected these changes without altering the production of the 3′-phosphoinositides PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 in the membrane (Figure 8), indicating that under certain circumstances, selected PH domains are retained in the cell interior despite the presence of a full complement of 3′-phosphoinositides. To test whether other methods of inducing ceramide accumulation also had these effects, NIH-3T3 cells were treated with PDMP, a glucosylceramide synthase inhibitor known to cause relatively small increases in ceramide accumulation without affecting PI-3K [36]. Under these conditions, PDMP also inhibited Akt/PKB phosphorylation (Figure 1). These studies suggest a partial mechanism by which ceramide inhibits PI-3K-dependent signalling by specifically preventing the recruitment of PH-domain containing proteins to membrane domains enriched in PtdIns(3,4,5)P_3.

Ceramide and PI-3K

Mounting evidence supports the idea that ceramide does not directly affect PI-3K. First, pretreatment of various cell types with C2-ceramide does not affect the amount of insulin-stimulated or growth-factor-stimulated PI-3K activity that co-immunoprecipitates with phosphotyrosine-containing proteins [22,23,25,26]. Secondly, the addition of C2-ceramide directly to isolated PI-3K does not alter its enzymic activity [44]. Nevertheless, the possibility remained that C2-ceramide could affect PtdIns(3,4,5)P_3 accumulation in a manner not previously observed. For example, C2-ceramide could activate a lipid phosphatase, such as SH2-containing 5-phosphatase ('SHIP') [45] or PTEN [46], to degrade the PtdIns(3,4,5)P_3 product rapidly. Alternatively, C2-ceramide could inhibit PI-3K activity indirectly through an intermediate that was washed away during isolation of the PI-3K enzyme. We therefore felt that it was important to measure PtdIns(3,4,5)P_3 levels directly and not to rely on the PI-3K assay as an indicator of PtdIns(3,4,5)P_3 accumulation. This was particularly important given the observed inhibition of GFP–Akt-PH and GFP–GRP-PH translocation, because these fusion proteins have been used as selective probes for the detection of cellular PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 [39]. By using a well-established assay for extracting and quantifying PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 levels, we determined that C2-ceramide did not affect the production of either phospholipid (Figure 8). Our results also indicate that cells lacking the PtdIns(3,4,5)P_3 phosphatase PTEN retained sensitivity to ceramide, indicating that the sphingolipid is unlikely to mediate its effects by targeting PTEN to a selected pool of PtdIns(3,4,5)P_3 (Figure 9).

Zundel et al. [47] previously reported that C2-ceramide inhibits basal PI-3K activity through the intermediate caveolin 1. Interestingly, in these and other studies [22,23,25,26], C2-ceramide apparently did not alter PI-3K kinase activity after stimulation with insulin or other growth factors. These results indicate that C2-ceramide inhibits the PI-3K pathway through different mechanisms; in unstimulated cells it affects PI-3K

through caveolin 1, whereas in stimulated cells it antagonizes PI-3K-mediated events by blocking the translocation of some or all of its downstream effector enzymes.

Ceramide and Akt/PKB translocation and phosphorylation

PI-3K stimulates Akt/PKB kinase activity by promoting its phosphorylation on two regulatory residues (Ser-473 and Thr-308). A serine/threonine kinase, PDK1 [48], phosphorylates the Thr-308 site of Akt/PKB after the recruitment of both proteins to the membrane by PtdIns(3,4,5)P_3 [11]. Phosphorylation of the second residue apparently results from autophosphorylation after the aforementioned Thr-308 phosphorylation [49] but the involvement of other upstream kinases has been proposed [50,51]. Several groups, including ours, have reported that C2-ceramide inhibits insulin-stimulated or growth-factor-stimulated phosphorylation of both the Ser-473 [22,25–27,47,52] and Thr-308 [24,26,44] phosphorylation sites. The results presented here are consistent with the hypothesis that C2-ceramide inhibits Akt/PKB phosphorylation by blocking its recruitment to the plasma membrane, thus preventing the phosphorylation reactions from occurring. This hypothesis is supported by similar results obtained by Watton and Downward [40], who discovered that ceramide prevents the translocation of the GFP-tagged Akt/PKB used here but did not evaluate the translocation of GFP–GRP1-PH or the accumulation of 3′-phosphoinositides. The results above, indicating that ceramide also inhibits the translocation of GRP1, suggest that the ceramide effect might generalize to many other PI-3K effectors. If so, ceramide could also inhibit the translocation of PDK1, which would further contribute to the ceramide-induced inhibition of Akt/PKB phosphorylation.

Surprisingly, two studies have indicated that in other cell types ceramide might regulate Akt/PKB by alternative mechanisms. First, Schubert et al. [27] reported that in TF-1 cells ceramide affects the Ser-473 site, but not the Thr-308 site, by activating a phosphatase sensitive to calyculin and okadaic acid. This seems to be in direct contrast with the various other cell types in which ceramide inhibits phosphorylation of both sites [24,26,44]. Moreover, if the current model for Thr-308 phosphorylation (i.e. phosphorylation follows translocation) is correct, the lack of inhibition of Thr-308 phosphorylation suggests that ceramide does not inhibit Akt/PKB translocation in these cells. Secondly, Salinas et al. [26] reported that in PC12 cells ceramide does not inhibit Akt/PKB translocation to the plasma membrane, although they did find that it inhibited its phosphorylation on both regulatory residues. They also reasoned that the effects of ceramide were mediated by a ceramide-activated protein phosphatase. The involvement of a specific phosphatase is not inconsistent with the results presented here; ceramide could prevent translocation and also activate a specific phosphatase in the NIH-3T3 cells used above. The most likely explanation for the results obtained in these other studies is that they reflect alternative means by which ceramide alters Akt/PKB-dependent signalling in other cell types.

Potential mechanisms

The most compelling finding above is that ceramide prevents the translocation of selected PH domains despite the lack of an effect on 3′-phosphoinositides. This indicates the existence of a novel mechanism underlying the regulation of selected PH-domain-containing proteins. If some target of ceramide disrupts interactions between the lipids and the PH domains, its identity is not readily apparent. Ceramide activates a protein phosphatase (ceramide-activated protein phosphatase, 'CAPP'), protein

kinase C-ζ (PKCζ) and the kinase suppressor of Ras (KSR). However, in our studies, inhibitors of CAPP and PKCζ had no effect on Akt/PKB phosphorylation [22]; neither did inhibitors of MEK (MAP kinase/ERK kinase) [44], which is activated downstream of KSR. Moreover, inhibitors of several other well-known signalling intermediates (i.e. protein kinase A, protein kinase C, protein kinase G and the mammalian target of rapamycin) also did not abolish the ceramide effect [22].

Instead of acting through an intermediate, the possibility remains that ceramide could interact directly with either the PH domains or the 3′-phosphoinositides. PH domains typically demonstrate a remarkable specificity for selected phospholipids [10]. Because ceramide or other ceramide derivatives do not structurally resemble the 3′-phosphoinositides, a direct competition between ceramide and 3′-phosphoinositides for binding on the PH domain seems unlikely. Nevertheless, this was the rationale for evaluating the effect of C1P because it, as a phosphorylated lipid, seemed most likely to resemble the phosphorylated 3′-phosphoinositides. Treatment of cells with C1P concentrations shown previously to increase its intracellular concentration markedly had no effect on Akt/PKB phosphorylation (Figure 1) but a direct interaction between ceramide itself or some other ceramide derivative with PtdIns(3,4,5)P_3-binding PH domains remains a possibility. The other possibility, that ceramide interacts with PtdIns(3,4,5)P_3, might also be considered. Alternatively, ceramide could localize to regions enriched in PtdIns(3,4,5)P_3, diluting its effective concentration in microdomains of the membrane and thus preventing Akt/PKB and GRP recruitment to the appropriate location. Because many signalling proteins and sphingolipid derivatives are localized in caveolae, we considered the possibility that ceramide might displace PIP$_3$ from caveolae. However, the cholesterol-binding reagent nystatin, under conditions known to disrupt caveolae formation completely, had no effect on PDGF-stimulated GFP–GRP-PH translocation or on ceramide's inhibition of this event (S. Stratford and S. A. Summers, unpublished work).

Implications

A staggering number of cellular stimuli transmit signals to the cell by acutely altering the cellular composition of membrane lipids. In particular, the phospholipid products of PI-3K, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, as well as the sphingolipid ceramide, have emerged as critical signalling intermediates that regulate a diverse array of biological processes. Recent studies reveal that these two signalling pathways also regulate one another. The activation of PI-3K causes a decrease in cellular ceramide levels [53], whereas ceramide inhibits PI-3K-dependent signalling [22,25–27,47,52]. The studies described here reveal that ceramide might act generally to prevent the translocation of PtdIns(3,4,5)P_3-binding proteins such as Akt/PKB and GRP1. Remarkably, ceramide blocked their translocation through a novel mechanism unrelated to the production of 3′-phosphoinositides. Collectively, these findings reveal the existence of another regulatory pathway that modulates PH-domain translocation. Furthermore, they suggest a general mechanism by which stimuli that induce ceramide synthesis could antagonize PI-3K-dependent signalling.

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