Phospholipase D activity is essential for actin localization and actin-based motility in *Dictyostelium*

Soha ZOUWAIL*, Trevor R. PETTITT†, Stephen K. DOVE*, Margarita V. CHIBALINA*[1], Dale J. POWNER†, Lee HAYNES†[2], Michael J. O. WAKELAM† and Robert H. INSALL*[3]

*School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K., and †Cancer Research UK Institute for Cancer Studies, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K.

PLD (phospholipase D) activity catalyses the generation of the lipid messenger phosphatidic acid, which has been implicated in a number of cellular processes, particularly the regulation of membrane traffic. In the present study, we report that disruption of PLD signalling causes unexpectedly profound effects on the actin-based motility of *Dictyostelium*. Cells in which PLD activity is inhibited by butan-1-ol show a complete loss of actin-based structures, accompanied by relocalization of F-actin into small clusters, and eventually the nucleus, without a visible fall in levels of F-actin. Addition of exogenous phosphatidic acid reverses the effects of butan-1-ol, confirming that these effects are caused by inhibition of PLD. Loss of motility correlates with complete inhibition of endocytosis and a reduction in phagocytosis. Inhibition of PLD caused a major decrease in the synthesis of PtdIns(4,5)P_2, which could again be reversed by exogenously applied phosphatidic acid. Thus the essential role of PLD signalling in both motility and endocytosis appears to be mediated directly via regulation of PtdIns(4)*P* kinase activity. This implies that localized PLD-regulated synthesis of PtdIns(4,5)P_2 is essential for *Dictyostelium* actin function.

Key words: actin polymerization, *Dictyostelium*, endocytosis, phospholipase D, phosphoinositide.

INTRODUCTION

PLD (phospholipase D) catalyses the hydrolysis of membrane phospholipids, such as PtdCho (phosphatidylcholine), producing the free headgroup and PtdOH (phosphatidic acid) (reviewed in [1,2]). PtdOH can be converted into diacylglycerol, which can function as a signalling molecule. However, it has been found recently that PtdOH can also be a signalling molecule in its own right. In particular, it has been proposed to regulate several aspects of intracellular vesicle traffic, as well as the activity of several signalling enzymes, including mTOR (mammalian target of rapamycin) [3], cAMP phosphodiesterases [4] and the tyrosine kinase Fgr [5]. It has also been implicated in the regulation of the actin cytoskeleton [6]. PLD has therefore emerged as an important signalling molecule, with roles in multiple aspects of cell biology. However, the pathways downstream of PLD are poorly understood.

Phospholipase D was first cloned from plants; the *Saccharomyces cerevisiae* SPO14 gene was identified as a PLD by homology with plant PLDs, and subsequently PLD genes have been identified in a range of mammals, *Drosophila* and *Caenorhabditis elegans* [1]. Each organism examined to date, with the exception of *Drosophila*, apparently has more than one PLD gene (M. J. O. Wakelam, unpublished work), with plants containing at least 12 genes [7]. Each PLD has two conserved HXKXXXXD domains and a SANIN amino acid sequence; these together are essential for catalytic activity.

PLDs catalyse a transphosphatidylation reaction in which water functions as a nucleophilic acceptor; short chain primary alcohols, such as ethanol or butan-1-ol, can function as stronger phosphatidyl group acceptors and thus a phosphatidylalcohol is preferentially generated. Phosphatidylalcohols are only produced by PLDs and are not normally detected in biological membranes (except in *S. cerevisiae*, which generate their own ethanol) [8]. Because of these properties, accumulation of phosphatidylalcohols [9,10] is diagnostic of PLD activity. At higher concentrations of primary alcohols, generation of phosphatidylalcohols dominates, so PLDs are unable to catalyse PtdOH production and signalling downstream of PLD is blocked. Because transphosphatidylation is specific for primary alcohols, similar levels of tertiary alcohols can be used as controls for non-specific toxicity.

PtdIns(4,5)P_2 generation is catalysed by the phosphorylation of PtdIns(4)*P* by a phosphoinositide-4-phosphate 5-kinase activity. In mammalian cells, the type I phosphoinositide-4-phosphate 5-kinases have been shown to be regulated by PtdOH; the type Iγ form, which is reported to be extensively activated by this signalling lipid, has been shown to interact with talin and is believed to regulate focal adhesions [9,10].

In the present study, we show that butan-1-ol is a specific and effective inhibitor of PLD activity in the social amoeba, *Dictyostelium discoideum*. Inhibiting PLD causes unexpectedly complete and specific inhibition of both actin-based motility and endocytosis. Previous work has suggested a connection between PLD and actin polymerization [6]. In *Dictyostelium*, however, we find that inhibition of PLD activity causes complete collapse of F-actin organization. Actin continues to polymerize, but at aberrant locations within the cell. We show further that this response is

Abbreviations used: ABD, actin-binding domain of ABP120; ARF, ADP-ribosylation factor; Arp2/3, actin-related protein 2/3; GAP, GTPase-activating protein; GFP, green fluorescent protein; mTOR, mammalian target of rapamycin; PLD, phospholipase D; PtdBut, phosphatidylbutanol; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdOH, phosphatidic acid; TRITC, tetramethylrhodamine β-isothiocyanate.

[1] Present address: Wellcome Trust Centre for the Study of Molecular Mechanisms in Disease, Cambridge Institute for Medical Research, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2XY, U.K.

[2] Present address: The Physiological Laboratory, University of Liverpool, Crown Street, Liverpool L69 3BX, U.K.

[3] To whom correspondence should be addressed (email R.H.Insall@bham.ac.uk).

mediated via a phosphoinositide-4-phosphate 5-kinase activity, implying a role for PLD-regulated synthesis of PtdIns(4,5)P_2 in normal *Dictyostelium* motility.

MATERIALS AND METHODS

Cell culture and development

Dictyostelium cells were grown axenically in HL-5 medium at 22 °C either in Petri dishes or shaken in flasks. For experiments requiring bacterially grown cells, *Dictyostelium* were plated on to lawns of *Klebsiella aerogenes* and harvested after 48 h by repeated washing in KK_2 buffer (16 mM potassium phosphate, pH 6.3).

Quantification of fluid-phase uptake

Measuring of fluid-phase uptake was performed as described by Maniak et al. [11]. Samples (10 ml) of 5×10^6 AX3 cells/ml were shaken in 25 ml flasks at 150 rev./min. TRITC (tetramethylrhodamine β-isothiocyanate)–dextran (M_r 70 000) (Sigma) was added to a final concentration of 2 mg/ml. Samples of 1 ml were withdrawn at intervals and added to 100 μl of Trypan Blue solution to quench extracellular fluorescence (2 mg/ml of Trypan Blue (Sigma) was prepared according to Hed [12], and passed through a 0.45-μm-pore-size Millipore filter). Samples were centrifuged for 3 min at 500 ***g***. The cell pellet was washed once in 1 ml of KK_2 buffer, resuspended in 1 ml of KK_2 buffer, and relative intensity was immediately measured in a PTI fluorescence spectrometer at 544 nm for excitation and 574 nm for emission.

Recording of ABD (actin-binding domain of ABP120)–GFP (green fluorescent protein) distributions and macropinosome formation

Transfected cells diluted to 5×10^5 cells/ml in nutrient medium were passed through Dvorak–Stotler chamber and were allowed to adhere to the glass coverslips for 30 min. Then fresh low fluorescent medium containing 1 mg/ml TRITC–dextran was passed through the chamber. Confocal sections were obtained using a Bio-Rad Radiance 2000 confocal on a Nikon 300 microscope by scanning at intervals of 15 s in one plane. In a single scan, GFP and TRITC were excited by a 488 nm argon-ion laser and a 543 nm HeNe laser respectively. Emitted light was collected with a 60× oil objective. Images were not processed further.

Immunofluorescence microscopy

Cells were allowed to adhere to glass coverslips for 30 min and then were fixed with picric acid/formaldehyde for 30 min. Actin filaments were stained using Texas Red–phalloidin (Molecular Probes). Nuclei were stained using Hoechst 33258 (Sigma). Cells were observed using a 63× Achroplan objective on a Zeiss Axioskop microscope. Images were captured using a Hamamatsu Orca camera driven by Improvision software, and were assembled using Adobe Photoshop, but were not processed further.

Cell speed

The mean speed of bacterially grown AX3 cells (which move more rapidly than axenic cells) was estimated using time-lapse recordings of phase-contrast images of the cells. Cells were washed free of bacteria, plated in KK_2 buffer containing 2 mM $MgCl_2$ on to glass-bottom culture plates, and allowed to adhere for 30 min. Phase-contrast images of the cells were obtained using a Zeiss Axiovert microscope with a 32× objective. Images were obtained every 15 s, and cell movement over a 10 min period was measured by plotting changes in the position of the centroid between frames, using a Macro written in the NIH (National Institutes of Health) Image program. The speed of an individual cell was calculated from the sum of the displacement in the 15 s interval between frames.

Phagocytosis assay

Phagocytosis of bacteria was performed as described elsewhere [13]. *Dictyostelium* cells were inoculated at a density of 10^6 cells/ml into a 20 ml suspension of *Escherichia coli* strain B/r at 10^9 cells/ml in KK_2 buffer. Culture (1 ml) was removed at regular intervals and the D_{600} was measured. Each assay was performed in triplicate, the means ± S.D. were plotted against time, and an estimate of phagocytic rate was calculated from the best-fit straight line.

In vivo labelling of phosphoinositides

Exponential-phase confluent cells were grown in low-phosphate medium, MES-HL5, for at least 12 h before culturing the cells in 10 mM Mes buffer (pH 6.5) for the period of the assay. Cells were labelled with 300 μCi/ml of [^{32}P]orthophosphate for 30 min. The alcohols were added to the shaking cells for another 30 min. Cells were washed free from label with 10 mM Mes buffer (pH 6.5), and PtdOH was added. Subsequent extraction, TLC, deacylation and HPLC analyses were exactly as described elsewhere [14].

Lipid analysis

The generation of [^{3}H]PtdBut (phosphatidylbutanol) utilized cells pre-labelled with 1 μCi/ml of [^{3}H]palmitate incubated with butanol for the times and concentrations stated. Lipids were extracted into chloroform/methanol as described previously [15], and the [^{3}H]PtdBut was separated by TLC before quantification by liquid-scintillation spectroscopy. The structural analysis of the non-radiolabelled lipids utilized chromatographic separation of total lipid extracts by HPLC on a Luna silica column linked to a Shimadzu QP8000alpha mass spectrometer. Detection in negative-ionization mode allowed identification of PtdBut, PtdOH, PtdIns, PtdEtn (phosphatidylethanolamine) and PtdSer (phosphatidylserine), while positive-ionization mode was used to detect PtdCho [16].

RESULTS

PLD activity is essential for normal cell motility

In preliminary experiments, we found that inhibition of PLD signalling diminished *Dictyostelium* cell growth in axenic medium or on bacterial plates (see the Supplementary Figure 1 at http://www.BiochemJ.org/bj/389/bj3890207add.htm). We therefore examined the mechanisms for this inhibition. First, we measured the effects of PLD inhibition on cell motility. Bacterially grown cells were transferred to glass-bottom plates and were observed by videomicroscopy, with images collected every 15 s (Supplementary Movie 1 at http://www.BiochemJ.org/bj/389/bj3890207add.htm). As shown in Figure 1(A), untreated cells moved at a rate of 6.3 ± 1.0 μm/min. When PLD activity was inhibited by 30 mM butan-1-ol, cells moved at two-thirds of this speed (4.1 ± 1.3 μm/min), while 70 mM butan-1-ol caused almost total immobility. Experiments of this type usually overestimate motility, because some changes in cell shape register as movement, so it is very unusual to see the movement of living cells blocked so completely. However, even after 30 min of immobility, washing the butan-1-ol out with fresh medium allowed the cells to recover normal motility (also see Supplementary Movie 4 at http://www.BiochemJ.org/bj/389/bj3890207add.htm).

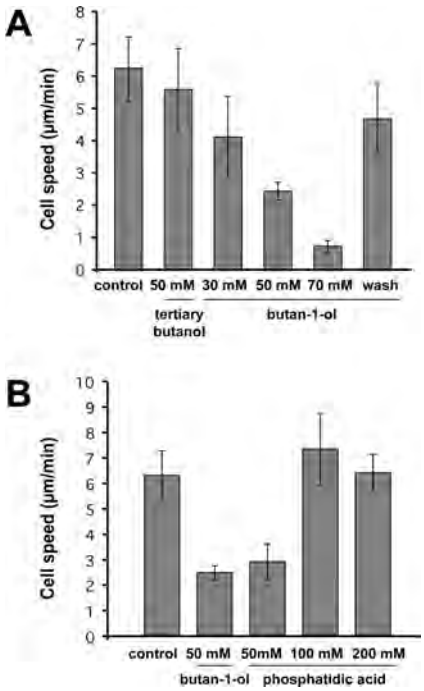

Figure 1 Effects of inhibition of PLD on cell movement

(**A**) Impaired cell movement in the presence of butan-1-ol. The speed of AX3 cells was calculated by analysis of time-lapse movies as described in the Materials and methods section. Speeds were measured 30 min after cells were treated with different concentrations of butan-1-ol and t-butanol. (**B**) Restoration of normal cell speed with PtdOH. The speed of AX3 cells was calculated by analysis of time-lapse movies as described in the Materials and methods section. Speeds were measured 5 min after the addition of PtdOH on butanol-1-ol-treated cells.

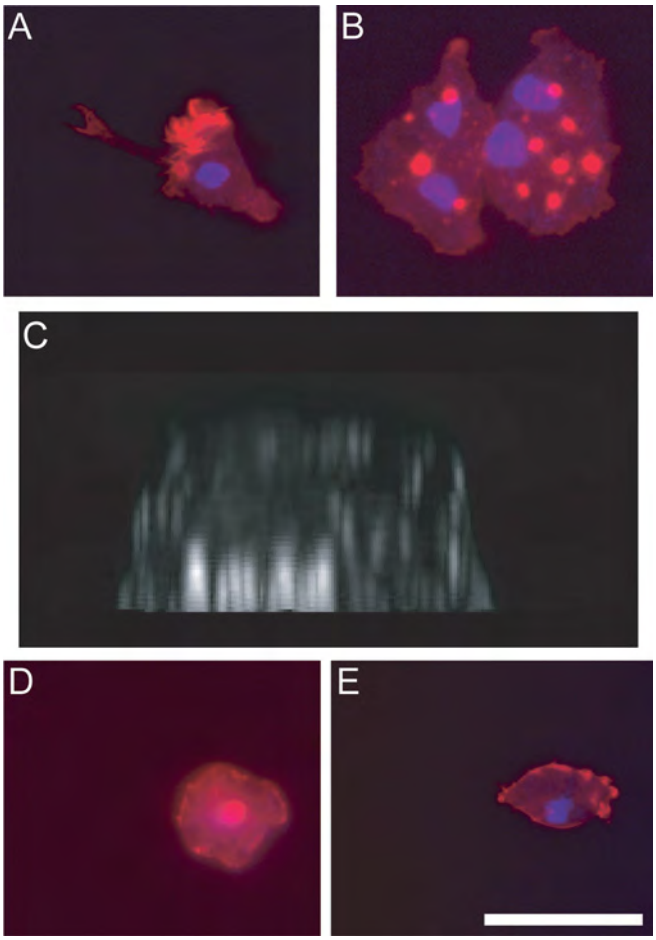

Figure 2 Redistribution of F-actin following PLD inhibition

Normal F-actin distribution in cells stained with Texas Red–phalloidin (red) and DAPI (4,6-diamino-2-phenylindole) (blue). AX3 cells (**A**), in the presence of 50 mM butan-1-ol (**B**), (**C**) and (**D**) and 50 mM t-butanol (**E**). In the absence of butanol, F-actin accumulated in macropinocytosis crowns and at the leading edge. This staining pattern was altered in the presence of 50 mM butanol, where F-actin accumulated in aggregates throughout the cells (**B**). A side view of a three-dimensional reconstruction of a similar cell (**C**) (one panel from Supplementary Movie 2 at http://www.BiochemJ.org/bj/389/bj3890207add.htm) shows that the largest aggregates are found at the base of the cells. After approx. 15 min, the F-actin is also seen to accumulate in the nucleus (**D**) (blue). Scale bar, 10 μM.

To confirm that these changes in cell speed were caused by inhibition of PtdOH formation through PLD, 100 μM dioctanoyl-PtdOH (a soluble and membrane-permeant analogue of PtdOH) was added to butan-1-ol-treated cells. This treatment restored normal cell speed within 5 min (Figure 1B), even after 30 min of butan-1-ol-induced immobility. The effects of butan-1-ol were therefore (i) specific to PLD activity, as replacing the product of the PLD enzyme abolished the inhibition, and (ii) fully reversible, suggesting that butan-1-ol was not non-specifically toxic. As a further control, we repeated all experiments with t-butanol, a similarly hydrophobic and membrane-permeant molecule: 50 mM t-butanol had no effect on cell movement. It therefore appears that PLD signalling is essential for *Dictyostelium* cell motility.

Inhibition of PLD activity causes aberrant F-actin distribution

Previous studies have implied a connection between PLD and regulation of actin polymerization (for example, see [6,17]). We therefore tested whether inhibition of PLD signalling by butan-1-ol resulted in actin defects. Cells were incubated in the presence of different concentrations of butan-1-ol, fixed and stained with Texas Red–phalloidin to visualize F-actin. The results were both unexpected and extremely clear: even in cells treated with 30 mM butan-1-ol, which only caused a partial inhibition of cell movement, F-actin was almost totally mislocalized within the cell. At 50 mM butan-1-ol, the effects were even more apparent. A short time after butan-1-ol was added, the majority of the F-actin was distributed in the form of clusters, which varied in size and were distributed throughout the cytoplasm and in cell cortex (Figure 2). Three-dimensional reconstruction of the cells with actin clusters, as shown in Figure 2(C) and Supplementary Movie 2 (see http://www.BiochemJ.org/bj/389/bj3890207add.htm), showed that the largest clusters were located at the bases of cells, although spots of F-actin could be seen all over the surface. A second striking feature was visible after longer incubations (15 min or more): in almost all of the cells that were treated with 50 mM, and many of the cells with 30 mM, butan-1-ol, F-actin was substantially relocalized to the nucleus (Figure 2D and Supplementary Movie 3 at http://www.BiochemJ.org/bj/389/bj3890207add.htm). t-Butanol did not cause either effect (Figure 2E and Supplementary Movie 5 at http://www.BiochemJ.org/bj/389/bj3890207add.htm).

As an alternative approach to studying F-actin dynamics, we observed live cells that were expressing the fluorescent F-actin marker ABD–GFP, which specifically binds F-actin, allowing the simultaneous visualization of actin dynamics and cellular behaviour in living cells [18]. Using confocal microscopy, it is possible to distinguish fluorescence of TRITC–dextran that has been taken up into vesicles from the fluorescence of the

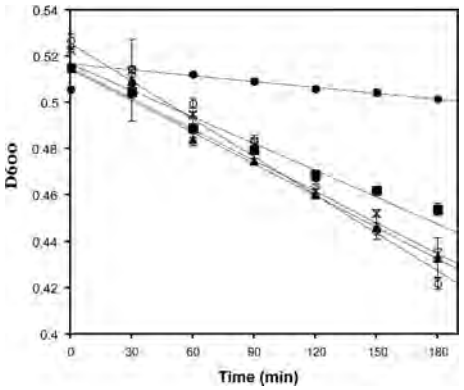

Figure 3 Disruption of PLD signalling reduces phagocytosis

AX3 cells were seeded into flasks containing a suspension of *E. coli* B/r in KK_2 buffer containing different concentrations of butan-1-ol or t-butanol. An aliquot of 1 ml from each culture was removed at various times, and the D_{600} was determined using a spectrophotometer. The graphs show the means ± S.D. for three independent cultures: the decrease in the density of the bacterial culture was taken as a measure of phagocytosis. ∗, No addition; ▲, 30 mM butan-1-ol; ■, 50 mM butan-1-ol; ●, 70 mM butan-1-ol; ○, 70 mM t-butanol.

external fluid [19]. We used this technique, with AX3 cells in a Dvorak–Stotler chamber, to show the time course of changes in F-actin localization after infusion of 50 mM butan-1-ol into the chamber. Supplementary Movie 3 (http://www.BiochemJ.org/bj/389/bj3890207add.htm) shows typically severe changes in actin distribution induced by the addition of 50 mM butan-1-ol. Within 3 min of butan-1-ol addition, we observed retraction of filopodia, loss of actin lamellae and near-total loss of cell polarity. These changes were accompanied by an obvious relocalization of F-actin into punctate aggregates of various sizes in the cell cortex, and also eventually into the nuclear region, reinforcing the results from the phalloidin staining in Figure 2(D).

As we had seen previously, the effects of butan-1-ol on actin were rapidly reversible. Supplementary Movie 4 (http://www.BiochemJ.org/bj/389/bj3890207add.htm) shows a typical experiment: within a few minutes of washing, cells recovered normal F-actin dynamics. Again the effects of butan-1-ol were specific for PLD activity. Supplementary Movie 5 (http://www.BiochemJ.org/bj/389/bj3890207add.htm) shows the minimal effects of 50 mM t-butanol on actin localization. Likewise, Supplementary Movie 6 (http://www.BiochemJ.org/bj/389/bj3890207add.htm) shows complete restoration of both macropinosomes and filopodia upon addition of dioctanoyl-PtdOH to butan-1-ol-treated cells. Minutes after dioctanoyl-PtdOH addition, cells begin making morphologically normal F-actin ruffles and macropinosomes. The effects of butan-1-ol on actin distribution and endocytosis are thus clearly and straightforwardly due to loss of PtdOH, presumably due to the inhibition of PLD signalling.

Disruption of PLD signalling reduces rates of phagocytosis and fluid-phase endocytosis

Existing work on PLD suggests a principal role in endocytosis (in *Dictyostelium*, mainly phagocytosis for bacterially grown cells and macropinocytosis for axenic cells) [2,8]. To determine the role of PLD in phagocytosis, wild-type cells were shaken with bacteria in the presence of different concentrations of butan-1-ol. Figure 3 shows that the rate of bacteria consumption was nearly halved by treating cells with 50 mM butan-1-ol, and was almost completely blocked in the presence of 70 mM butan-1-ol. This effect is specific for PLD: Figure 3 shows no difference in phagocytosis rates in the presence of 70 mM t-butanol.

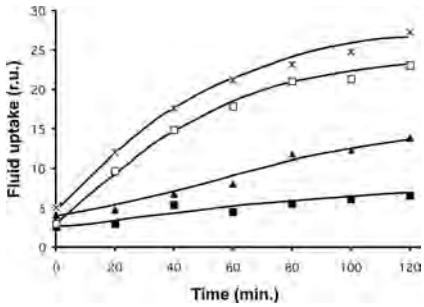

Figure 4 Decreased endocytosis following inhibition of PLD

AX3 cells were shaken in the presence of TRITC–dextran. An aliquot of 1 ml of culture was removed at various times, washed, and the amount of internalized medium was determined using a fluorimeter. The results represent the mean of four separate experiments, measured in relative fluorescence units (r.u.). ∗, No addition; ▲, 30 mM butan-1-ol; ■, 50 mM butan-1-ol; □, 50 mM t-butanol.

Much of the uptake of fluid phase that occurs during axenic growth in *Dictyostelium* is through the actin-based process of macropinocytosis [19]. We assessed the effects of PLD inhibition on macropinocytosis by shaking cells in axenic medium containing TRITC–dextran and different concentrations of butan-1-ol. Samples were taken at various intervals and washed, and the quantity of internalized fluorescent medium determined [19]. As shown in Figure 4, the rate of uptake of fluorescent medium was decreased by 80 % in the presence of 50 mM butan-1-ol, whereas the same concentration of t-butanol caused very little change, indicating that macropinocytosis was also almost entirely inhibited when PLD signalling was blocked. This clearly implies that a PLD activity is required for both fluid-phase endocytosis and phagocytosis.

For a more detailed examination of fluid-phase endocytosis in living cells, we observed cells transfected with ABD–GFP as described above. Figures 5(a)–5(c) shows the process of macropinosome formation in living cells. Untreated cells take up medium by extending actin-rich protrusions (arrowheads), which then enclose a bubble of extracellular fluid. The formation of macropinosomes was abolished completely within 3 min of treatment with 50 mM butan-1-ol (Figures 5d–5f). This process is also clearly visible in Supplementary Movie 3 (http://www.BiochemJ.org/bj/389/bj3890207add.htm): macropinosome formation, like pseudopod assembly, was inhibited completely by butan-1-ol. This inhibition of endocytosis, like the redistribution of actin, was rapidly reversible: washing out the butanol allowed cells to restart macropinocytosis within 5 min (Figures 5g–5o). Supplementary Movie 4 (http://www.BiochemJ.org/bj/389/bj3890207add.htm) emphasizes this point further. Unexpectedly, after butan-1-ol was washed away, an unusually high number of macropinosomes was formed, which suggests that some activator of macropinocytosis had accumulated which was dependent upon PLD signalling for its action.

A butanol-sensitive PLD activity in *Dictyostelium*

The reversal of the effects of butan-1-ol by exogenous PtdOH confirms that the inhibition of actin dynamics is due to a PLD activity. To characterize this activity further, we examined PtdBut formation. Figure 6 shows that PtdBut was generated in the cells exposed to increasing concentrations of butan-1-ol. The quantity of PtdBut formed in *Dictyostelium* was lower than that found in butan-1-ol-treated mammalian cells, but the dose–response relationship was similar, and in particular was consistent with that necessary for inhibition of actin dynamics, motility and endocytosis, with approx. 30 mM butan-1-ol being required for

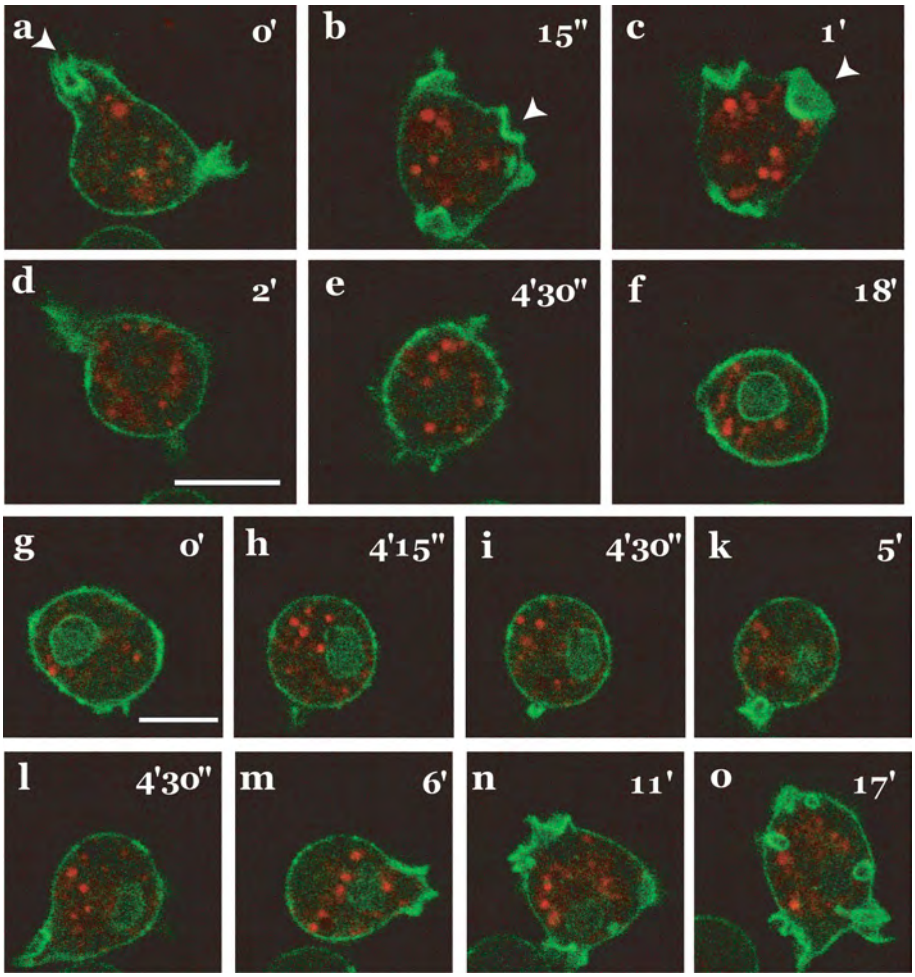

Figure 5 Loss of macropinosomes following inhibition of PLD

Confocal sections of live ABD–GFP-transfected cells, which had been pre-treated with TRITC–dextran to show endosomes, taken at the indicated times. (**a**)–(**c**) Normal macropinosome formation in untreated cells; (**d**)–(**f**) loss of macropinosomes and aberrant actin distribution when medium with 50 mM butan-1-ol was added to the same cell at 1 min; (**g**)–(**k**) the same cell immediately after fresh medium was added; (**l**)–(**o**) recovery of motility, normal actin distribution and macropinosome formation in fresh medium. All images are panels taken from Supplementary Movies 3 and 4 (http://www.BiochemJ.org/bj/389/bj3890207add.htm). Scale bar, 10 μM.

almost maximal PtdBut formation. We therefore conclude that 30 mM butan-1-ol is sufficient to give near-complete inhibition of pathways downstream of PLD, with somewhat more needed for complete abrogation of PLD-mediated PtdOH production.

HPLC–MS analysis of the PtdBut species yielded unexpected results (Figure 6B). The principal PtdBut species formed (approx. 65 %) were ether lipids, the remaining 35 % being diacyl species. As we have observed when analysing PtdBut species in yeast and mammalian cells, the lipids were primarily mono-unsaturated, with some di-unsaturated species being detected. This is in keeping with our previous proposal that PtdOH species with this degree of unsaturation are the signalling forms [16]. Ether lipids are usually found as PtdEtns rather than PtdChos. This suggests that the PLD activities in *Dictyostelium* may be more similar to plant PLDs, which are more selective for PtdEtn, rather than the mammalian PLDs, which typically catalyse PtdCho hydrolysis.

Loss of PLD activity causes inhibition of PtdIns(4,5)P_2 synthesis

Different inositol lipids have separate and essential roles in the control of the actin cytoskeleton. In the light of the severity of actin phenotypes shown above, we therefore examined phosphoinositide synthesis following butan-1-ol treatment. Cells were labelled for 30 min with [^{32}P]orthophosphate and were treated with butan-1-ol, after which lipids were extracted and separated by TLC and HPLC. Under these very short-term conditions, ^{32}P incorporation into lipids reflects PtdIns(4,5)P_2 synthesis, not overall levels of PtdIns(4,5)P_2, which require many hours of incubation with ^{32}P to become significantly radiolabelled. Inhibition of PLD activity by butan-1-ol caused a major decrease in the rate of incorporation of ^{32}P into PtdIns(4,5)P_2 (Figure 7). When butan-1-ol was washed away, the rate of PtdIns(4,5)P_2 labelling returned to normal (and indeed consistently exceeded the resting rate, again suggesting that a co-factor or substrate was accumulating in butan-1-ol-inhibited cells). The effect of butan-1-ol could be reversed by the addition of exogenous PtdOH, while t-butanol did not cause any inhibition, again confirming that this effect was specifically caused by inhibition of PLD. It is important to note that the assay shown in Figure 7 only shows new synthesis of PtdIns(4,5)P_2, not overall levels (which were not significantly affected by the short-term treatments described here; results not shown). Inhibition of phosphoinositide-4-phosphate 5-kinase activity on these timescales would prevent localized increases in PtdIns(4,5)P_2, rather than causing an overall depletion.

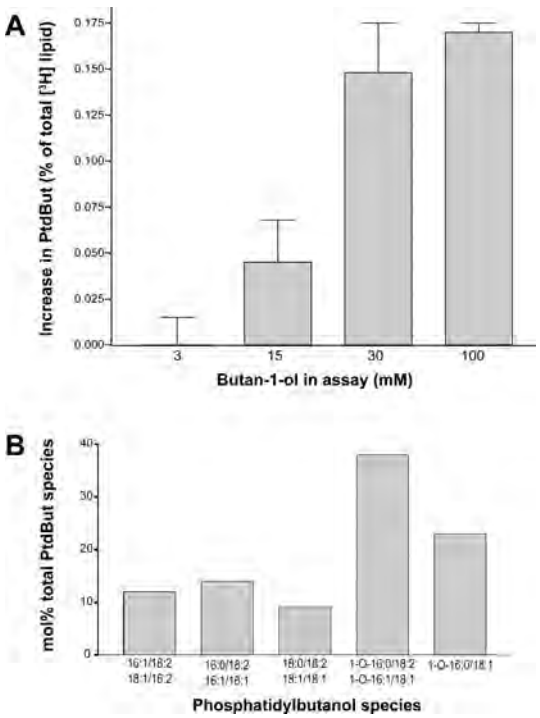

Figure 6 Generation of phosphatidylbutanol

(**A**) [^{3}H]palmitate-labelled AX3 cells were incubated with increasing concentrations of butan-1-ol for 5 min before lipid extraction and analysis of [^{3}H]PtdBut formation. (**B**) AX3 cells were incubated in the presence of 50 mM butan-1-ol for 5 min, the lipids were extracted and the PtdBut species were identified by LC-MS.

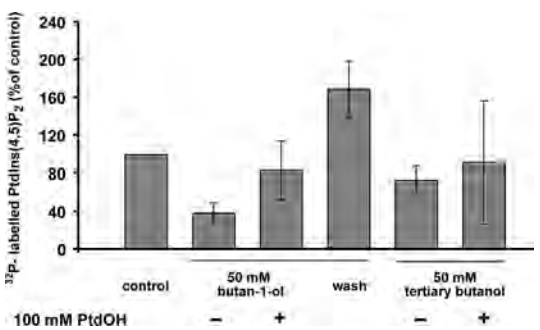

Figure 7 Butan-1-ol decreases incorporation of ^{32}P into PtdIns(4,5)P_2

Cells were labelled for 30 min with [^{32}P]orthophosphate and were treated with butan-1-ol, after which lipids were extracted and separated by TLC and HPLC.

It therefore appears that PLD activity is required for the majority of phosphoinositide-4-phosphate 5-kinase activity in *Dictyostelium* cells *in vivo*. This is a surprising result: there are a large number of genes encoding phosphoinositide-4-phosphate 5-kinase homologues, and regulation by PtdOH has only been previously observed *in vitro* in a small subset of phosphoinositide-4-phosphate 5-kinase isoforms [9,20,21]. PtdIns(4,5)P_2 synthesis is known to be required for both normal actin vesicle traffic and function. It is therefore most likely that the target for PtdOH generated by PLD is one or more phosphoinositide-4-phosphate 5-kinases which are required for normal actin dynamics and endocytosis.

DISCUSSION

The results presented in this paper demonstrate essential roles for PLD activity in the regulation of multiple aspects of *Dictyostelium* cell physiology. We have made use of the ability of primary alcohols, such as butan-1-ol, to act as preferential nucleophilic acceptors in the PLD-catalysed transphosphatidylation reaction, and have thus blocked the formation of PtdOH. Multiple controls show that butan-1-ol acts specifically as an inhibitor of PLD signalling. We have also shown that inhibition of PLD causes an unexpectedly severe decrease in phosphoinositide-4-phosphate 5-kinase activity, providing a mechanism by which PLD most likely exerts its effects. The simplest model is that PtdOH is a second messenger which is required for phosphoinositide-4-phosphate 5-kinase activity, and that localized synthesis of PtdIns(4,5)P_2 is essential for normal F-actin localization.

The clearest requirements for PLD activity are in the control of actin dynamics and endocytosis. A number of previous studies have shown the involvement of PLD in both of these processes. However, our results are novel in a number of respects. Firstly, the inhibition of motility (in particular) that we see is both more rapid and far more complete than seen in other work, such as the wound-healing response of Madin–Darby canine kidney cells [22]. Secondly, previous work has shown that inhibition of PLD decreases actin polymerization. In butan-1-ol-treated *Dictyostelium*, the obvious change is a total relocalization of the F-actin, first into punctate clusters around the cell perimeter, and later within the nucleus. A major function of PLD in *Dictyostelium* would therefore appear to be in the localization, not activation, of actin-nucleating factors such as the Arp2/3 (actin-related protein 2/3) complex. The factors that localize actin polymerization are not well understood, but are the subject of great current interest. A number of studies have suggested that PtdIns(4,5)P_2 itself plays an important role in both activation and localization of actin nucleation factors, though the details remain controversial. One possible explanation for our data is that the main role of PtdIns(4,5)P_2 in *Dictyostelium* is to localize actin polymerization to pseudopods and macropinosomes; in the absence of PtdIns(4,5)P_2 synthesis, other less efficient signals may cause actin to assemble in puncta and in the nucleus. It is important to note that the rapid inhibition by butan-1-ol is not consistent with an overall drop in PtdIns(4,5)P_2 levels, which would take several hours of inhibition. Rather, normal actin polymerization must require localized activation of phosphoinositide-4-phosphate 5-kinase to synthesize small amounts of PtdIns(4,5)P_2 in a restricted location, such as a vesicle or a lipid domain.

The nuclear localization of F-actin following prolonged butan-1-ol treatment is particularly interesting. The nuclear F-actin shown in Figures 2 and 5 is plainly polymerized normally, since it binds to both phalloidin and ABD–GFP, although, to our surprise, it appears not to contain Arp3 (results not shown) and neither therefore the Arp2/3 complex. Reports of nuclear F-actin have occasionally appeared, although few dependable data have emerged concerning either its distribution or function. Mammalian and *Dictyostelium* cells both relocalize F-actin to the nucleus following DMSO treatment [23,24], but the relevance of this process is still not clear. The nuclear signal we see could reflect a physiological actin localization, which is normally masked by the large amount of actin polymerization in the cortex. Alternatively, nuclear actin polymerization could have been caused by the increased levels of free actin monomers when normal actin polymerization is blocked.

We have used a number of approaches to demonstrate that butan-1-ol was acting as a specific inhibitor of PLD signalling, rather than non-specifically inhibiting cell function. t-Butanol had

little or none of the same inhibitory activities, while addition of exogenous PtdOH rescued all phenotypes observed. Figure 6(A) shows that the butan-1-ol dose-dependence of PtdBut formation was similar to that observed in mammalian, plant and yeast cells, and importantly was also similar to that for the inhibition of cellular function, pointing to a key role for PLD-generated PtdOH in regulating cellular events [15]. Figure 6(A) also shows that the PLD reaction product in *Dictyostelium* is primarily mono-unsaturated, as we have observed previously for both mammalian [16] and yeast [8] cells, which points to this being the signalling form and also suggests a common structure for PtdOH-binding domains between organisms. Nevertheless, in contrast with mammalian and yeast cells, the *Dictyostelium* PLD primarily hydrolyses ether phospholipids (Figure 6B). Since ether lipids are mainly PtdEtns, the data suggest that the *Dictyostelium* enzyme hydrolyses this lipid, although it remains possible that a minor alkyl pool of PtdCho is the substrate. The plant PLDs have been reported to hydrolyse PtdIns, PtdCho and PtdEtn [7], thus the *Dictyostelium* enzymes appear to have more in common with these than the yeast or mammalian forms. Database searches reveal three different *Dictyostelium* homologues of PLD1 [registered in Dictybase (http://dictybase.org/) as *pld*A, *pld*B and *pld*C; and S. Zouwail and R. H. Insall, unpublished work], but these are about equally similar to both plant and mammalian enzymes.

These data point to the generated PtdOH functioning as a second messenger. While it is possible that PLD-catalysed PtdCho or PtdEtn hydrolysis could induce cellular effects by modifying membrane structure, it would be surprising if different lipid families were used to achieve the same architectural changes in mammals and *Dictyostelium*. In mammalian cells, it has been demonstrated that PtdOH can regulate the activity of the tyrosine kinase Fgr [5], $p47^{phox}$ [25], protein phosphatase 1γ [26], phosphoinositide-4-phosphate 5-kinases $I\alpha$ and $I\gamma$, the ARF (ADP-ribosylation factor) GAPs (GTPase-activating proteins) ASAP1 (ARF-GAP containing Src homology 3 domain, ankyrin repeats and pleckstrin homology domains 1) [27] and AGAP1 (ARF-GAP containing GTP-binding-protein-like, ankyrin repeat and pleckstrin homology domains 1) [28], the cAMP phosphodiesterase 4A1 [4] and mTOR [3]. In addition, PtdOH binding to Raf1 [29], ARF1, ARF6, β-cop coatamer, NSF and kinesin [30] suggests further regulatory effects. The PtdOH targets to date all have roles in regulating membrane trafficking, actin cytoskeletal changes and potentially, in the case of mTOR, in cell proliferation, thus it may be that *Dictyostelium* homologues of these proteins are also regulated by PtdOH, explaining the regulation of endocytosis, phagocytosis and migration by PLD in this organism.

We are very grateful to Dr David Knecht (University of Connecticut) for the ABD–GFP construct and for helpful advice on microscopy. The work in this paper was funded by a component grant from the MRC (Medical Research Council), and a programme grant from the Wellcome Trust. R. H. I. is funded by an MRC Senior Fellowship. The authors have no competing commercial interests.

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Received 12 January 2005/14 March 2005; accepted 15 March 2005
Published as BJ Immediate Publication 15 March 2005, DOI 10.1042/BJ20050085