Characterization of phospholipase activity in *Dictyostelium discoideum*

Identification of a Ca^{2+} -dependent polyphosphoinositidase-specific phospholipase C

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We have identified a Ca^{2+} -dependent polyphosphoinositide-specific phospholipase C activity in *Dictyostelium discoideum*. Addition of Ca^{2+} ($20\ \mu\text{M}$) results in the rapid formation of $\text{Ins}(1,4,5)\text{P}_3$ within 5 s and leads to sustained inositol phosphate production for up to 40 min in membranes prepared from [^3H]inositol-labelled cells. The phospholipase C activity is primarily membrane-bound under the conditions used to lyse the cells. In addition to this activity we also identified a family of Ca^{2+} -regulated phospholipase activities active on a range of phospholipid substrates, using [^3H]palmitate labelling. Inositol-specific phospholipase C activity is highest in vegetatively growing cells and in starved cells during the first 6 h in development, during which time Ca^{2+} elicited a 5-fold stimulation of inositol phosphate formation. After this time, total activity decreased progressively until 15 h, after which the activity remained constant up until 24 h. During this period, Ca^{2+} was able to stimulate a 2-fold increase in inositol phosphates.

INTRODUCTION

In the cellular slime mould *Dictyostelium discoideum*, starvation leads to the development of a multicellular organism that involves the chemotactic aggregation of $\sim 10^5$ cells to form a multicellular aggregate and the continued differentiation that results in the formation of a mature fruiting body containing a sorocarp on top of a vacuolated stalk (for reviews see [1–3]). Although the central role of cyclic AMP acting through G-protein-coupled cell-surface receptors in co-ordinating multicellular development is now well documented, the signal-transduction processes activated in response to cyclic AMP receptor occupation remain relatively poorly understood. Recent technological advances have led to the rapid identification of genes encoding multiple cyclic AMP receptor subtypes [4,5] and an even greater number of G-protein α -subunits present during development ([6–8]; C. Gaskins & R. A. Firtel, unpublished work), implying that progression through development proceeds via a complex interplay of a number of second-messenger systems. These advances have themselves led to significant insights into the regulation of gene expression in *Dictyostelium*, but are only the first stage in developing a complete understanding of how *Dictyostelium* development is co-ordinated. The next stage in expanding this knowledge is to determine which second-messenger systems are activated in response to each receptor subtype, and at what stages in development they function.

Recent results have identified at least three second-messenger systems activated by cyclic AMP via G-protein-coupled pathways in *Dictyostelium*: adenylate cyclase [9,10], guanylate cyclase [11,12] and inositol phospholipid/ Ca^{2+} messenger systems that have been shown to be essential in regulating gene expression, as well as chemotaxis and morphogenesis [13–16]. Although all three systems have been demonstrated to be activated by extracellular cyclic AMP bound to cell-surface receptors and guanosine 5'-[γ -thio]triphosphate (GTP[S]) in membranes or permeabilized cell preparations [17–19], the $G\alpha$ subunits with which they interact, and the times in development when they are active,

have not yet been identified. In addition to these pathways, other signal-transduction mechanisms, for example mediated by folic acid or cell–cell contact, exist, but remain poorly understood.

As an initial step to increase our understanding of the role of individual second-messenger systems, we have begun characterizing the inositol-lipid signal-transduction pathway and have investigated the potential role of other lipid-based signal-transduction pathways. The inositol lipid/diacylglycerol/ Ca^{2+} signalling system is not yet well characterized in these cells, and in fact direct measurement of intracellular Ca^{2+} levels in *Dictyostelium* has proved difficult when using the intracellular fluorescent indicators quin 2 and fura 2 [20]. Furthermore, inositol lipid and phosphate measurements have been hampered by the poor uptake and complex metabolism of [^3H]inositol. These difficulties arise from differences in inositol metabolism in *Dictyostelium* compared with mammalian cells and are caused primarily from the existence of significant inositol kinase activity, and additional enzymes that are capable of forming relatively large amounts of InsP_6 directly from inositol [19–23]. The production of these metabolites of inositol complicate inositol phosphate analysis, because their presence overwhelms the receptor-mediated formation of inositol phosphates and because it is difficult to differentiate (even by h.p.l.c.), in whole cells, between the two mechanisms of formation. In addition, the relatively unusual lipid composition [24,25] and the absence of C_{20} unsaturated fatty acids such as arachidonic acid [26] imply that there may be other differences in inositol lipid metabolism in terms of diacylglycerol production and utilization.

In many mammalian cell types, second-messenger signal transduction can occur by other mechanisms in addition to those already identified in *Dictyostelium*. For example, receptor-mediated activation of phospholipase A_2 activity [27] and phosphatidylcholine-specific phospholipase C and D activities [28–30] are present in many cell types and play important roles in cellular regulation. However, these activities have not been examined in detail in *Dictyostelium discoideum*. We have examined phospholipase activities present in *Dictyostelium* and have

quantified their relative specific activities during development. In order to do this, we have modified standard electroporation protocols to introduce [^3H]inositol efficiently into cells and to measure inositol phosphate formation free from high levels of endogenous soluble [^3H]inositol-labelled compounds. In addition, we have used [^3H]palmitate to label the major phospholipids and neutral lipids in *Dictyostelium* to determine if they undergo phospholipase A-mediated hydrolysis. We find that there is a Ca^{2+} -activated membrane-bound $\text{PtdIns}(4,5)\text{P}_2$ -specific phospholipase C activity in *Dictyostelium* and that its activity is greatest in vegetative cells and in early development. After 6 h of development, at the mid-aggregate stage, its activity falls progressively until 15 h, at which time its activity remains constant at approx. 30% of the original level throughout culmination. Furthermore, we have identified a family of Ca^{2+} -activated phospholipase activities that are active against a range of phospholipids.

MATERIALS AND METHODS

Growth and development of *Dictyostelium*

Strain KAx-3 was grown axenically in HL-5 medium in shaking culture or on plastic surfaces in Petri dishes, as described previously [31,32]. Cells were washed and plated for development as described in the above references.

Electroporation of cells

Cells were electroporated by a method based on those of Howard *et al.* [33] and Van Haastert *et al.* [19] with modification. Exponentially growing cells were washed twice in electroporation buffer (50 mM-Mes, pH 6.1, 1.0 mM-EGTA at 4 °C) and resuspended at a density of 2×10^8 cells/ml in this buffer also containing [^3H]inositol. All subsequent steps were carried out on ice in precooled cuvettes. Unless stated otherwise, [^3H]inositol was added at a concentration of 50 $\mu\text{Ci}/\text{ml}$. Cells were electroporated in 0.44 ml batches with two 800 V pulses separated by a 5 s interval, by using a 5 Ω resistor, 3 μF capacitor and with a 0.2 cm electrode gap, with a Bio-Rad Gene Pulser electroporation apparatus. Initial experiments were carried out over a range of voltages to optimize the incorporation of inositol into cellular membranes. After electroporation, 4 μl of 100 $\times$ recovery solution (100 mM- CaCl_2 /100 mM- MgCl_2) was added and the cells were left on ice for at least 5 min. After this time, the cells were carefully removed from the cuvettes and added to fresh HL-5 medium at room temperature at a final density of 4×10^6 cells/ml and placed on a rotating platform overnight (12–18 h). Labelled cells were then processed as described under phospholipase C assay below.

Labelling of cells with [^3H]palmitate

Exponentially growing cells were centrifuged and washed in fresh HL-5 medium, resuspended at a density of 4.8×10^7 cells/ml in 25 ml, and 0.2 mCi of [^3H]palmitate was added directly to the cells. After brief mixing, the suspension was added to more HL-5 medium to give a final density of 4×10^6 cells/ml. Cells were incubated with [^3H]palmitate overnight, a time previously found to give optimal uptake of the label into all phospholipid classes (results not shown). Labelled cells were then processed as described under phospholipase C assay below.

Phospholipase assay

Labelled cells were washed twice in wash buffer (20 mM- NaCl , 20 mM- KCl , 10 mM-Mes, pH 6.2, 0.1 mM- CaCl_2 , 4 °C) and resuspended in homogenization buffer (10 mM-Hepes, pH 7.5, 1 mM-EGTA, 1 mM-dithiothreitol, 0.2 mM-phenylmethanesulphonyl

fluoride, 0.2 mM-leupeptin, 11 μg of chymostatin/ml, 8 μg of pepstatin/ml, 6 μg of antipain/ml and 2 μg of aprotinin/ml). Resuspended cells were lysed by two passages through a 5 μm Nucleopore membrane precooled to 4 °C, and 5 $\times$ assay buffer was added to give (final concns.) 100 mM- KCl , 25 mM-Hepes, pH 7.0, 6 mM- MgCl_2 , 5 mM-ATP, 1 mM-dithiothreitol, 1 mg of BSA/ml, 10 mM-phosphocreatine, 10 units of creatine kinase/ml and 2 mM-EGTA. Free Ca^{2+} concentrations were calculated by using a computer program [34] with Ca^{2+} /EGTA buffers. In most experiments 10 mM- LiCl or -2,3-bisphosphoglycerate was also included to inhibit inositol phosphate breakdown. In experiments where GTP[S] was used, the free Ca^{2+} concentration was buffered to 40 nM. Lysed cells were centrifuged (18 500 g for 17 min) at 4 °C in a 'Tomy' micro-centrifuge. Membranes were resuspended in assay buffer at 4 °C equivalent to an original cell concentration of 6.7×10^6 cells/ml in 0.2 ml and added to test tubes with or without drugs for 20 min at 25 °C.

For studies of inositol lipid analysis and inositol phosphate formation by ion-exchange column chromatography, reactions were terminated by the addition of 1 ml of chloroform/methanol/conc. HCl (100:100:1, by vol.). Phases were split by addition of 0.1 ml of 10 mM-EDTA, and, after mixing and brief centrifugation to separate the phases, the upper phase was transferred to a separate tube. The lower phase was then washed with 1 ml of pre-equilibrated upper phase, and the upper phase was combined with the previous wash. The lower phase was then used for analysis of inositol lipids as described for the palmitate-labelled lipids, and the upper phase, after drying down under air and dilution with water to 5 ml, was used for inositol phosphate analysis on ion-exchange columns as described below.

For palmitate-labelled cells, lipids were extracted by addition of 1 ml of chloroform/methanol/conc. HCl (100:100:1, by vol.) as above and separated from precipitated protein by centrifugation. The protein pellet was then washed with 1 ml of pre-equilibrated lower phase and the washings were transferred to the first extraction. Solvent was then evaporated under nitrogen, and the samples were resuspended and plated in chloroform/methanol/water (100:100:1, by vol.).

Lipid analysis

Neutral lipids were analysed by sequential one-dimensional t.l.c. on Whatman LK6D silica gel 60 plates, based on the method of MacDonald & Weeks [35]. Plates were first developed in isopropyl ether/acetic acid (99:1, v/v) and dried in air. Complete separation of neutral lipid classes was then performed by development in light petroleum (b.p. 35–60 °C)/diethyl ether/acetic acid (90:10:1, by vol.).

Phospholipids were analysed by t.l.c. by using Whatman LK6D silica 60 plates either sequentially in one dimension [36] or in two dimensions on Whatman K6 silica 60 plates [37]. In both cases, plates were developed first in chloroform/methanol/4 M- NH_3 also containing 2 mM-cyclohexylenedinitrilotetra-acetic acid (9:7:2, by vol.), and after air drying and rotation through 90° (for two-dimensional plates) then developed in chloroform/methanol/acetic acid/water (25:15:4:2, by vol.). Lipid spots were identified by comparison with authentic standards that were made visible by exposure to iodine vapour. After allowing the iodine to sublime, the spots were scraped into scintillation vials and, after addition of 4 ml of Liquescent (National Diagnostics), radioactivity was counted in a Beckman scintillation counter.

H.p.l.c. analysis

H.p.l.c. analysis was carried out based on the method of Cantley and co-workers [38]. Samples were quenched by addition of 0.6 ml of ice-cold HClO_4 (3.16%, w/v) and, after brief

centrifugation to remove precipitated protein, the samples were neutralized by addition of 0.2 ml of 2 M-KOH/0.1 M-Mes/25 mM-EDTA and left on ice for 5 min. After this time, samples were centrifuged to pellet KClO_4 , and the supernatant was transferred to separate tubes. Samples were stored frozen at -70°C before analysis by h.p.l.c. Samples (0.4 ml) were separated by h.p.l.c. on a 5 μm Partisphere SAX column (Phenomenex) (25 cm $\times$ 0.46 cm) with a discontinuous gradient up to 1 M-(NH_4)₂HPO₄ buffered to pH 3.8 with H_3PO_4 and at a flow rate of 1.0 ml/min. Before sample addition the h.p.l.c. column was equilibrated with water, and after sample addition the column was washed with water for 10 min. Subsequently the concentration of ammonium phosphate was increased to 0.25 M over 1 h, and then to 1 M over 45 min. This concentration was then maintained for 10 min before returning to 100% water over 10 min. Water was run through the column for at least 30 min before the next sample analysis. Fractions (0.5 ml) were collected, starting 5 min after sample injection, for 100 min. After addition of 0.5 ml of methanol and 4 ml of Liquescent, radioactivity was measured by liquid-scintillation counting.

Inositol phosphate analysis by Dowex columns

Inositol phosphates were separated into lower (InsP_1 , InsP_2 , InsP_3 and InsP_4) and higher (InsP_5 and InsP_6) inositol phosphates fractions by using Bio-Rad Ag1 X8 resin (200–400 mesh; Cl^- form). The Cl^- resin provides better resolution of higher inositol phosphates than the formate form and is more readily regenerated [39]. Samples were loaded in 5 ml on to 2.9 cm $\times$ 0.8 ml columns and washed with 40 ml of water to remove inositol. The lower inositol phosphates (InsP_1 , InsP_2 , InsP_3 and InsP_4) were collected together with elution with 10 ml of 325 mM-HCl, whereas the higher inositol phosphates InsP_5 and InsP_6 were collected separately, by elution with 10 ml of 1.5 M-HCl. This method results in less than 5% contamination of each fraction [39].

Materials

Lipids were from Avanti Polar Lipids (Birmingham, AL, U.S.A.). [^3H]Inositol was from Amersham, and [^3H]palmitate was from NEN. H.p.l.c.-grade solvents were from J. T. Baker. Biochemical reagents were from Boehringer Mannheim. T.l.c. plates were from Whatman. [^3H]Ins1P and [^3H]Ins(1,4) P_2 h.p.l.c. standards were a gift from Dr. P. T. Taylor (University of California, San Diego). [^3H]Ins(1,4,5) P_3 standard was from Amersham. The [^3H]glyceroinositol phosphate standard was prepared from [^3H]PtdIns after separation by t.l.c. and deacylation as described in [38].

RESULTS

Analysis of [^3H]inositol-labelled cells

[^3H]Inositol was readily incorporated into cells by using modifications of electroporation protocols reported previously (see the Materials and methods section). In our hands, [^3H]inositol uptake and incorporation into lipids was most efficient at relatively low voltages and could be further improved by overnight incubation in fresh medium. Under these conditions, $\sim 5\%$ of the added [^3H]inositol was taken up by cells (results not shown). Because of the difficulties of identifying Ins(1,4,5) P_3 formation in whole cells, we undertook to examine inositol phosphate production in membranes prepared from [^3H]inositol-labelled cells. By this approach, it was possible to eliminate completely potential contamination of the Ins(1,4,5) P_3 h.p.l.c. peak by other inositol triphosphates normally present in the cytosol. In initial experiments, the labelled membranes were resuspended in cytosol prepared from identically treated but

unlabelled cells. This approach was designed to provide soluble phospholipase C activity or other components, but to prevent the addition of high levels of [^3H]inositol phosphates formed during the labelling incubation time that would interfere with the detection of newly formed inositol phosphates. Initial studies revealed a significant Ca^{2+} -stimulated activity with identical characteristics at 0, 3 and 6 h of development. The results from experiments using membranes and cytosol prepared from vegetative cells are shown in Fig. 1; Ca^{2+} stimulated a dose-dependent increase in inositol phosphate formation, resulting in an approx. 5-fold increase in inositol phosphates produced over the incubation time relative to control incubations (see the Materials and methods section). The half-maximal stimulation of inositol phosphate production occurred at a free Ca^{2+} concentration of 0.2 μM . [^3H]Inositol phosphates were fractionated into lower (InsP_1 , InsP_2 , InsP_3 and InsP_4) and higher (InsP_5 and InsP_6) inositol phosphates on ion-exchange columns. Results indicated that increases in [^3H]inositol phosphates were confined to the lower inositol phosphates. Higher inositol phosphates (InsP_5 and InsP_6), although present in significant amounts, displayed no Ca^{2+} -dependent increases (results not shown). As shown in Fig. 1(b), there was a corresponding decrease in [^3H]inositol lipids.

In order to determine what fraction of phospholipase C activity was membrane-bound under the experimental conditions employed, experiments were done with membrane fractions in the complete absence of added cytosol or in the presence of various amounts of cytosol. The results from this experiment demonstrated that addition of cytosol had little ($< 15\%$) effect on inositol phosphate formation, implying that most of the phospholipase C activity is membrane-bound (results not shown). Subsequent experiments were therefore carried out in the absence of added cytosol, and virtually identical results were obtained under these conditions.

Ca^{2+} -stimulated inositol phosphate production was seen within 5 s (see Fig. 2) and was sustained for up to 40 min in the presence of 10 mM-LiCl. In membranes prepared from vegetative cells [^3H]inositol phosphate production continued to increase in a bimodal fashion up to 40 min, with $\sim \frac{1}{3}$ of the activity reaching a plateau after ~ 2 min. The reason for these kinetics is not known, but it may be due to the rapid depletion of a relatively small inositol lipid pool that is rapidly hydrolysed and then reformed at a slower rate, or the incomplete inhibition of inositol phosphate degradation by Li^+ . Other possibilities include the presence of two distinct enzymic activities with different kinetics, two pools of substrate that are hydrolysed at different rates, or a single activity that is activated through two different mechanisms.

H.p.l.c. analysis of inositol phosphate formation

To determine which inositol phosphates are formed during Ca^{2+} -stimulated activation, inositol phosphates were analysed by h.p.l.c. The results from these experiments are shown in Fig. 3. Ca^{2+} stimulated a rapid increase in Ins(1,4,5) P_3 and Ins(1,4) P_2 . Both inositol phosphates increased within 5 s of Ca^{2+} addition, whereas inositol monophosphate and glyceroinositol phosphate levels remained unchanged. This pattern of inositol phosphate formation became more complex as time increased, and led to the formation of additional inositol bisphosphate peaks and a second peak that was eluted behind Ins(1,4,5) P_3 . The failure to observe the formation of glyceroinositol phosphate suggests that Ca^{2+} does not activate a membrane-bound Ca^{2+} -dependent inositol-lipid-specific phospholipase A activity. The rapid sustained production of Ins(1,4,5) P_3 throughout the time course of the experiment demonstrates, by contrast, that Ca^{2+} activates a polyphosphoinositide-specific phospholipase C activity. The delayed accumulation of inositol monophosphate is consistent with

Fig. 1. Dose-dependency of Ca^{2+} regulation of $[^3\text{H}]$ inositol phosphate formation in membranes of *Dictyostelium*

Vegetative cells were labelled with $[^3\text{H}]$ inositol by electroporation and membranes prepared as described in the Materials and methods section. Membranes were resuspended in cytosol prepared from identically treated but unlabelled cells and incubated with various concentrations of Ca^{2+} for 20 min at 25°C . Reactions were terminated by the addition of 1 ml of ice-cold chloroform/methanol/HCl, and inositol phosphates (a) and total inositol lipid fractions (b) were separated and measured as described in the text. Results represent means $\pm$ S.D. of triplicate determinations. Symbols without error bars represent points in which the S.D. was smaller than the size of the symbols. Data represent typical results from an experiment repeated independently four times.

its formation either from hydrolysis of $\text{Ins}(1,4,5)\text{P}_3$ or $\text{Ins}(1,4)\text{P}_2$ or from a slower and less active hydrolysis of PtdIns .

Developmental kinetics of phospholipase C activity

In order to determine the potential developmental role of the inositol-lipid-specific phospholipase activity, Ca^{2+} -stimulated phospholipase C activity was measured throughout development (Fig. 4). The results from these experiments demonstrate that the Ca^{2+} -activated inositol phosphate formation is maximal in vegetative cells and that its activity steadily declines until approx. 15 h, after which its activity remains relatively constant until complete development. During this time, $[^3\text{H}]$ inositol lipid levels decrease by approx. 20% (results not shown), suggesting that decreases in inositol lipid radioactivity alone cannot account for this effect. In terms of fold increase, Ca^{2+} -activated inositol phosphate formation remains constant for the first 6 h of

Fig. 2. Time course of inositol phosphate formation in the presence of a maximal concentration of Ca^{2+}

Cells were labelled with $[^3\text{H}]$ inositol by electroporation and membranes prepared as described in the Materials and methods section. Membranes were resuspended in assay buffer and incubated at a free Ca^{2+} concentration of $20 \mu\text{M}$ ($\bullet$) or in EGTA alone ($\circ$) for the times shown. Reactions were terminated by the addition of 1 ml of ice-cold chloroform/methanol/HCl, and inositol phosphates were separated and measured as described in the text. Results represent the means $\pm$ S.D. of triplicate determinations. Symbols without error bars represent points in which the S.D. was smaller than the size of the symbols. Data represent typical results from an experiment repeated independently four times. Insert shows time course of inositol phosphate formation over relatively short time periods.

development, but falls dramatically between 6 and 15 h to remain constant after this time.

Analysis of $[^3\text{H}]$ palmitate-labelled lipids

In order to characterize phospholipase activities in *Dictyostelium* further, cells were labelled with $[^3\text{H}]$ palmitate overnight. Palmitate was chosen for the incorporation studies because it is a major component of the bacteria which are the normal food source of *Dictyostelium* and because its metabolism in *Dictyostelium* has already been relatively well characterized [40]. $[^3\text{H}]$ Palmitate is rapidly taken up into cellular lipids and readily desaturated and incorporated well into all of the C_{16} and C_{18} fatty acids. The conversion into C_{18} acids occurs by addition of a C_2 unit to the carboxyl end of palmitate without the break-up of the palmitate molecule. In these studies, we have used the ability of $[^3\text{H}]$ palmitate to label *Dictyostelium* lipids evenly, to determine if Ca^{2+} or GTP[S] can stimulate phospholipid degradation of classes of phospholipid other than the inositol lipids and to determine if this degradation occurs by a phospholipase A- or C-mediated mechanism. To approach this question, experiments were carried out under identical experimental conditions in membranes of $[^3\text{H}]$ palmitate-labelled cells, and both neutral-lipid formation and phospholipid degradation were examined.

Neutral-lipid analysis revealed a Ca^{2+} -dependent stimulation of neutral-lipid formation and a slight stimulation in response to GTP[S] (see Fig. 5). A GTP[S] -stimulated increase in triacylglycerols was observed at 40 nM free Ca^{2+} , but was relatively small and did not display dose-dependent characteristics consistent with the activation of a G-protein, which would be expected to show peak activation between 1 and $10 \mu\text{M}$ - GTP[S] [41] (Fig. 5b). Progressively higher concentrations of Ca^{2+} , by contrast, stimulated a dose-dependent increase in

Fig. 3. H.p.l.c. analysis of [^3H]inositol phosphate accumulation in the presence of a maximal dose of Ca^{2+} in membranes

Vegetative cells were labelled with [^3H]inositol by electroporation and membranes prepared as described in the Materials and methods section. Membranes were resuspended in assay buffer and incubated in the presence of $20\ \mu\text{M}$ free Ca^{2+} , or EGTA alone (control), at 25°C for the times indicated. Reactions were terminated by addition of $600\ \mu\text{l}$ of 3.16% HClO_4 , processed and run on a h.p.l.c. column as described in the Materials and methods section. Results represent typical h.p.l.c. runs of duplicate samples of an experiment repeated independently twice. The positions of authentic standards are indicated by arrows (GroInsP, glyceroinositol phosphate).

triacylglycerol and small increase in free palmitate at low Ca^{2+} concentrations, but failed to stimulate a significant increase in diacylglycerol over the 20 min incubation period (Fig. 5a). Although the magnitude of Ca^{2+} -stimulated formation of ^3H -labelled neutral lipids was considerably less than that of inositol phosphate accumulation, the Ca^{2+} -dependency was similar. The production of triacylglycerol under these conditions is somewhat surprising, since phospholipase A-, C- or D-mediated breakdown of phospholipids alone would not lead to the direct formation of this lipid. However, triacylglycerol could be formed by the Ca^{2+} -stimulated accumulation of both palmitic acid (via phospholipase A) and diacylglycerol (via phospholipase C), followed by subsequent acylation of the diacylglycerol with palmitate to form triacylglycerol. In fact, this interpretation is most consistent with the data, since triacylglycerol levels do not increase at the expense of either diacylglycerol or palmitate, implying that both are being constantly replenished. It is not possible, however, to differentiate between the two activities, except to conclude that Ca^{2+} activates both enzymes and the reacylation pathway coordinately.

To determine which phospholipids are broken down, phospholipids were analysed by two-dimensional t.l.c. The results

from these studies revealed that most classes of phospholipid undergo Ca^{2+} -activated hydrolysis (see Fig. 6). The greatest relative decreases in palmitate labelling occurred in cardiolipin and phosphatidylethanolamine, which were labelled to the greatest extent under these conditions. In addition to these changes, PtdIns and PtdSer levels also displayed smaller changes, and PtdCho decreased only slightly. Since palmitate labelling of phospholipids would reflect changes caused by both phospholipase A and C activities, this experiment cannot differentiate between the relative magnitudes of the two. These results imply, however, that there is a family of membrane-bound Ca^{2+} -activated phospholipase activities present in *Dictyostelium* capable of hydrolysing most cellular phospholipids.

DISCUSSION

These results demonstrate the existence of a Ca^{2+} -activated phospholipase C activity present in *Dictyostelium* membranes. Although the existence of phospholipase C activity in *Dictyostelium* has been inferred from the production of inositol phosphates formed in response to cyclic AMP in whole cells, this is the first demonstration, using endogenously labelled substrate,

Fig. 4. Developmental regulation of inositol-lipid-specific phospholipase C

Vegetative cells were labelled with [3 H]inositol by electroporation and, after recovery and washing, plated for development as described in the Materials and methods section. At appropriate times in development cells were washed from plates and membranes were prepared as described in the Materials and methods section. Membranes were resuspended in assay buffer and incubated in the presence of 20 μ M free Ca^{2+} or EGTA alone (control) for 20 min at 25 $^{\circ}\text{C}$. Reactions were terminated by addition of 1 ml of ice-cold chloroform/methanol/HCl, and lipids were separated and measured as described in the text. Results represent the means $\pm$ S.D. of triplicate determinations. Symbols without error bars represent points in which the S.D. was smaller than the size of the symbols. Data represent typical results from an experiment repeated independently three times. Insert represents Ca^{2+} -stimulated activity expressed as fold increase over basal.

of such an activity in the membranes of these cells. In the present study, we were able to measure significant (5-fold) and sustained (up to 40 min) increases in inositol phosphate formation stimulated by Ca^{2+} in the presence of Li^+ . Surprisingly, however, in initial experiments we have been unable to observe a GTP[S]-stimulated increase in inositol lipid hydrolysis in membranes, even under a variety of conditions. Phospholipase activities from a variety of organisms have now been purified and cloned, resulting in the identification of a number of distinct classes of phospholipase genes [42]. In previous studies in which the regulation of phospholipase C activity by Ca^{2+} in mammalian cells [43–45] and *Dictyostelium* [46] has been examined, it was found that Ca^{2+} concentrations over the physiological range stimulate the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$, whereas higher Ca^{2+} concentrations result in PtdIns hydrolysis. In most mammalian systems, Ca^{2+} (within the physiological range) acts synergistically with GTP[S] to enhance receptor-mediated inositol phosphate formation. Our results showing the activation of phospholipase C and generation of $\text{Ins}(1,4,5)\text{P}_3$ by Ca^{2+} concentrations within the physiological range are therefore consistent with this trend. In most of the mammalian cell types examined, it is clear that G-protein-mediated activation, and not Ca^{2+} , is responsible for the initial activation of phospholipase C. This also appears to be true in *Dictyostelium*, since cyclic AMP, acting through its cell surface receptors, stimulates InsP_3 production in wild-type *Dictyostelium* cells but not in $\text{g}\alpha 2$ null cells (K. Okaichi, A. B. Cubitt & R. A. Firtel, unpublished work). However, increased intracellular Ca^{2+} concentrations probably enhance phospholipase C activity in mammalian cells during sustained stimulations, since there is little G-protein-mediated activation of phospholipase C

Fig. 5. Effects of Ca^{2+} (a) and GTP[S] (b) on formation of [3 H]palmitate-labelled neutral lipid

Vegetative cells were labelled with [3 H]palmitate and membranes prepared as described in the Materials and methods section. Membranes were resuspended in assay buffer and incubated with various concentrations of Ca^{2+} for 20 min at 25 $^{\circ}\text{C}$. Reactions were terminated by addition of 1 ml of ice-cold chloroform/methanol/HCl, and neutral lipids were separated and measured as described in the text. Results represent the means $\pm$ S.D. of triplicate determinations. Symbols without error bars represent points in which the S.D. was smaller than the size of the symbols. Data represent typical results from an experiment repeated independently four times. Symbols: Δ , triacylglycerol; $\circ$, diacylglycerol; $\square$, palmitate.

in the complete absence of Ca^{2+} [44]. The situation in *Dictyostelium*, however, is unclear, because direct measurements of intracellular Ca^{2+} concentrations in this cell type have proved difficult to obtain. However, both the cyclic AMP and folic acid receptors have been demonstrated to activate Ca^{2+} channels directly [47], raising the possibility that Ca^{2+} may play a direct role in regulating phospholipid turnover in these cells.

Although previous studies have identified a GTP[S]-stimulated increase in $\text{Ins}(1,4,5)\text{P}_3$ in permeabilized cells [19], we were unable to detect this in our membrane preparations in the present study. This may indicate that G-protein-mediated activation of phospholipase C in *Dictyostelium* is very sensitive to conditions of cell homogenization and membrane preparation. Alternatively, GTP[S] may cause a rapid adaptation of the system similar to that seen in whole cells in response to receptor activation by cyclic AMP, making GTP[S]-stimulated activity difficult to measure. The existence of relatively high levels of Ca^{2+} -activated phospholipase C activity and sustained production of $\text{Ins}(1,4,5)\text{P}_3$ demonstrate that this activity is stable and that $\text{PtdIns}(4,5)\text{P}_2$ is present and available to be hydrolysed in membranes prepared under these conditions. Although it is possible that phospholipase C activity is regulated *in vivo* by Ca^{2+} , the published kinetics of Ca^{2+} influx [47] and inositol phosphate formation [19] suggest that Ca^{2+} alone could not

Fig. 6. Effect of Ca^{2+} on $[^3\text{H}]$ palmitate-labelled phospholipids

Cells were labelled with $[^3\text{H}]$ palmitate and membranes prepared as described in the Materials and methods section. Membranes were resuspended in assay buffer and incubated in the presence of $20\ \mu\text{M}$ free Ca^{2+} (■) or EGTA alone (▨; control) for 20 min at 25°C . Reactions were terminated by addition of 1 ml of ice-cold chloroform/methanol/HCl, and neutral lipids were separated and measured as described in the text. Results represent the means $\pm$ S.D. of triplicate determinations. Data represent typical results from an experiment repeated independently four times. Abbreviations: PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; PtdOH, phosphatidic acid.

account for the observed increase in inositol phosphates. Nevertheless, measurements of $\text{Ins}(1,4,5)\text{P}_3$ levels in whole cells (particularly in the absence of Li^+) may be a poor reflection of total inositol lipid turnover, because it is not possible to determine if the transient appearance of $\text{Ins}(1,4,5)\text{P}_3$ is due to a transient activation of phospholipase C or to its rapid metabolism in the cell. Measurements in *Dictyostelium* are further complicated by the difficulty of measuring $\text{Ins}(1,4,5)\text{P}_3$ levels free from a high background of highly labelled inositol metabolites which have similar mobilities on h.p.l.c. strong anion-exchange (SAX) columns. Attempts to identify a G-protein involvement in phospholipase C activation require the demonstration that the G-protein is necessary for phospholipase C activation *in vivo* and *in vitro*.

In addition to its activation of an inositol-lipid-specific phospholipase C, we also demonstrated that Ca^{2+} stimulated a range of phospholipase activities active against most phospholipids present in *Dictyostelium*. Previous studies [48] have also noted high levels of phospholipase A activity in *Dictyostelium*, consistent with the present study, although our results have shown in addition that there is a rapid re-incorporation of degraded fatty acids into triacylglycerol. It seems likely that differences in experimental conditions, such as the omission of ATP, addition of exogenous substrate and longer incubation time account for the inability of earlier studies to identify reacylation of fatty acids formed by phospholipase A action. Previous studies have demonstrated that cyclic-AMP-mediated induction of pre-spore and pre-stalk-enriched gene expression may be mediated in part by rises in $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol (see [1,2,15,16]). It is possible that activation of these phospholipase activities may also contribute to the regulation of these responses by increasing

diacylglycerol levels. In addition, *Dictyostelium* undergoes relatively dramatic changes in phospholipid composition, and decreases its total lipid content during the first 12 h of development [49–51]. It is possible that increases in intracellular Ca^{2+} concentration mediated by cyclic AMP could play a role in this process via the activation of phospholipase activity. Although cyclic AMP receptor occupation probably does not stimulate prolonged increases in intracellular Ca^{2+} concentrations, because of its relatively rapid adaptation [52], it is likely to cause transient increases in intracellular Ca^{2+} levels. Phospholipid remodelling could then occur by a series of progressive transient increases in intracellular Ca^{2+} concentration. Direct proof of this idea must wait until we are able to measure, in much greater detail, the changes in intracellular Ca^{2+} concentration and phospholipase activity during development.

H.p.l.c. analysis revealed the rapid formation of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,4)\text{P}_2$ in membranes of *Dictyostelium* in the presence of Ca^{2+} . Both inositol phosphates were stimulated within 5 s of activation and increased at similar rates. The magnitude of increase was greatest for $\text{Ins}(1,4)\text{P}_2$, and this may reflect the rapid degradation of $\text{Ins}(1,4,5)\text{P}_3$ to $\text{Ins}(1,4)\text{P}_2$ under these conditions, or the direct formation of $\text{Ins}(1,4)\text{P}_2$ from $\text{PtdIns}4\text{P}$. By comparison, $\text{Ins}4\text{P}$ accumulation occurred only after a lag and increased only gradually. A similar pattern of formation occurred with a second peak that was eluted close to $\text{Ins}(1,4)\text{P}_2$. The position of this peak is consistent with the relative mobility of $\text{Ins}(4,5)\text{P}_2$, and the formation of this compound would be in agreement with the known metabolism of $\text{Ins}(1,4,5)\text{P}_3$ in these cells [53]. In *Dictyostelium*, most of the $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase activity is membrane-bound, whereas most of the $\text{Ins}(1,4,5)\text{P}_3$ 1-phosphatase is soluble at this stage of development [53,54], implying that in membranes the major product of $\text{Ins}(1,4,5)\text{P}_3$ metabolism would be $\text{Ins}(1,4)\text{P}_2$, and that $\text{Ins}(4,5)\text{P}_2$ formation would be decreased. There was virtually no glyceroinositol phosphate formation throughout the time course of the experiment (2 min). Since this would be the water-soluble product of phospholipase A-mediated hydrolysis of PtdIns , this result clearly demonstrates that there is little Ca^{2+} -activated membrane-bound phospholipase A activity directed against PtdIns . Previous studies have demonstrated the existence of a soluble phospholipase A activity capable of hydrolysing exogenously added PtdIns in *Dictyostelium* [55], although the substrate specificity or activity towards membrane-bound lipids remains unknown. We have not at present examined the activity of cytosolic phospholipase activities directed against inositol lipids in *Dictyostelium*, but such studies using exogenously labelled substrates could be performed.

In conclusion, we have identified an inositol-lipid-specific phospholipase C activity in *Dictyostelium* and demonstrated that maximal levels of activity are expressed in vegetative cells and for the first 6 h of development. Although these results suggest that inositol lipid hydrolysis may play a greater role in growing cells and at early stages of development, it is worth noting that in the present study total Ca^{2+} -activated enzyme activity and not receptor-mediated activity was measured. Further work is necessary in order to understand more completely the role of inositol lipid hydrolysis in *Dictyostelium*.

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