Phosphatidic acid mobilized by phospholipase D is involved in the phorbol 12-myristate 13-acetate-induced G₂ delay of A431 cells

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This study was aimed at gaining an understanding of metabolic events responsible for the inhibition of cells in G₂ phase, a known physiological restriction site in the cell cycle of multicellular organisms. In an earlier study, phosphatidic acid was proposed as an inhibitory mediator in the epidermal growth factor (EGF)-induced inhibition of A431 cells in G₂ phase via the phospholipase C pathway [Kaszkin, Richards and Kinzel (1992) *Cancer Res.* 52, 5627–5634]. We show here that the phorbol ester phorbol 12-myristate 13-acetate (PMA) induces a reversible inhibition of the G₂/M transition in A431 cells under conditions of phospholipase D-catalysed phosphatidic acid formation. Such PMA-induced inhibition in G₂ phase is largely attenuated in the presence of 1-propanol (but not of 2-propanol). In this case the amount of phosphatidic acid is reduced to almost control levels, and instead

phosphatidylpropanol is formed. In the case of EGF-induced activation of a phospholipase D the amount of phosphatidic acid is only slightly decreased in the presence of a primary alcohol. Under these conditions the EGF-induced G₂ delay was not affected. The correlation between the formation of phosphatidic acid and the G₂ delay induced by PMA, as well as by an exogenous bacterial phospholipase D (from *Streptomyces chromofuscus*), could be supported by using synchronized cells in order to increase the population of cells in G₂ phase. This study indicates that the formation of substantial amounts of phosphatidic acid immediately before entry into mitosis seems to be important for establishing a delay in the cell cycle at the G₂/M border by exogenous ligands.

INTRODUCTION

The G₂ phase represents (in addition to the G₀ phase) a second physiological restriction site in the cell cycle of a large number of cells including germ cells as well as somatic cells [1,2]. Evidence from studies of cell tissue kinetics has indicated that a certain portion of cells rests physiologically in the G₂ phase; these cells are prepared to divide upon cell loss in the immediate neighbourhood [3]. Resumption of the mitotic process in such cases would presumably be triggered by extracellular signals. It is to be expected that the elements of the extracellular environment involved in growth control and intracellular growth regulators are tightly linked. Yet a physiological mechanism by which an extracellularly induced restriction of cells in G₂ phase may be elicited is unknown.

We present here what may be a model system for the study of at least one such mechanism in more detail. The extracellular ligands epidermal growth factor (EGF) and phorbol ester phorbol 12-myristate 13-acetate (PMA), each working via a different physiological signal pathway, have been shown to inhibit the G₂/M transition of a number of cultured cells in a reversible manner [4,5] as part of their bimodal function in growth control [6]. The restriction in G₂ phase by both ligands occurs so rapidly that a search for inhibitory mediator(s) was a promising approach and has already been started. In these studies phosphatidic acid turned out to be a possible candidate for such an inhibitory mediator in HeLa and A431 cells [7,8]. In recent years increasing evidence has been accumulated that phosphatidic acid may have biological functions of its own in a number of ligand-induced processes in vertebrate cells, particularly in growth control [9–13].

In ligand-induced changes of phospholipid metabolism two

major metabolic routes have been recognized as responsible for the rapid mobilization of phosphatidic acid. One is via activation of phospholipase C and conversion of the product by diacylglycerol kinase to phosphatidic acid; the other is by direct activation of phospholipase D (for reviews see [14–17]). In order to test the potential role of phosphatidic acid as a mediator of the ligand-induced inhibition of the G₂/M transition, both pathways of phosphatidic acid formation could be employed as tests. For this purpose we attempted to modulate the degree of ligand-induced phosphatidic acid formation and to study the consequences for inhibition of the G₂/M transition. Such attempts are, of course, limited to those interactions which themselves do not interfere to any great extent either with the cell-cycle progress or with morphological criteria on which the analysis of the G₂/M transition is based.

In an earlier study in A431 cells the EGF-induced formation of phosphatidic acid via phospholipase C and diacylglycerol kinase was tested with regard to its potential role in the G₂/M transition [8]. Part of the experiments was devoted to the manipulation of the formation of phosphatidic acid through modulation of the EGF receptor. The data obtained supported an inhibitory role for phosphatidic acid at the G₂/M transition.

In this study we have attempted to manipulate the second route of phosphatidic acid formation; namely, that via phospholipase D. It has been shown previously that bacterial phospholipase D is able to induce an inhibition of the G₂/M transition in A431 cells [8]. For the purpose of this study conditions have been determined under which the phorbol ester PMA leads to an increase of phosphatidic acid in A431 cells almost exclusively via activation of phospholipase D. Under these circumstances PMA is shown to induce an inhibition of the G₂/M transition. In view of the lack of inhibitors specific for

Abbreviations used: ARF, ADP-ribosylation factor; DMEM, Dulbecco's modified minimal essential medium; EGF, epidermal growth factor; PIP-kinase, phosphatidylinositol-4-phosphate 5-kinase; PIP₂, phosphatidylinositol 4,5-bisphosphate; PMA, phorbol 12-myristate 13-acetate.

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phospholipase D, the potential of phospholipase D to transfer phosphatidate to primary alcohols [18–20] was utilized in order to manipulate the amount of phosphatidic acid generated. This transphosphatidyl transfer reaction operates under physiological conditions in intact cells, and leads to a reduction of phosphatidic acid level with concomitant formation of phosphatidylalcohol. Under conditions where an increase in the amount of phosphatidic acid could not be reduced significantly, e.g. by treatment of cells with EGF in the presence of 1-propanol or by secondary alcohols, the induction of the G_2 delay was not influenced. Similar results obtained in cell cultures enriched in G_2 phase by synchronization seem to support the hypothesis that phosphatidic acid formed in substantial amounts acts as an inhibitory mediator of ligand-induced cell-cycle alterations at the G_2/M border.

MATERIALS AND METHODS

Materials

PMA, dioleoyl phosphatidic acid, 1,2- and 1,3-dioleoylglycerol, phospholipids and phospholipase D from *Streptomyces* sp. (Sigma Type VII) were obtained from Sigma (Munich, Germany). [$1-^{14}C$]Arachidonic acid (2.2 MBq/mmol) and [$1-^{14}C$]oleic acid (2.2 MBq/mmol) were obtained from Amersham Buchler (Braunschweig, Germany). Phospholipase D from *Streptomyces chromofuscus* was provided by Boehringer (Mannheim, Germany).

Cell culture

The epidermoid carcinoma cell line A431 (generously given by Professor H. zur Hausen, German Cancer Research Centre, Heidelberg, Germany) was cultivated routinely as monolayers in Dulbecco's modified minimal essential medium (DMEM) containing 10% (v/v) calf serum.

Single cell analysis of the transition from G_2 phase to mitosis by time-lapse photography

For time-lapse analysis of the G_2/M transition exponentially growing cells were established in plastic flasks. After addition of individual compounds, the flasks were closed tightly and placed under an inverted phase-contrast microscope at 37 °C. The details of the analysis of the G_2/M transition monitored by time-lapse photography have been described elsewhere [4,8]. The analysis consists in principle of a single-cell analysis on the basis of serial photographs taken every 10 min from the same field containing about 700–1000 cells and a 'retrospective' determination of the transition from G_2 phase to prophase of dividing cells. For the purpose of the correlation with biochemical data the number of cells entering prophase are presented in two ways: (1) as the number of cells entering prophase per 10 min period (which is equal to the G_2/M transition rate) seen in individual experiments; and (2) in cumulative form, in order to estimate the duration of the G_2 delay (for details see [4,5]). The cumulative data are presented for each compound as the sum of at least two experiments. For reasons discussed previously [4] in some experiments the G_2 delay was determined at the level $\log(N/N_0) \times 10^2 = 1$; N_0 = number of cells at zero time and N = number of cells at the given time.

Synchronization of A431 cell cultures

In order to confirm the results obtained in logarithmically growing cell cultures a set of experiments was carried out with synchronized cells, thereby obtaining a higher portion of cells in

Table 1 Percentage distribution of synchronized and logarithmically growing A431 cell cultures in different cell-cycle phases

The values for the cell populations in G_1 -, S- and G_2/M -phase were obtained by flow cytometry. The values for the mitotic figures were calculated from pictures of a parallel time-lapse-photography analysis. Abbreviations: AA-block, amethopterin/adenosine-block; a.r., after release; log-culture, logarithmically growing culture.

	G_1	S	G_2/M	Mitotic figures
AA-block	72	23	5	0
+ 7 h a.r.	8	30	62	10
+ 8 h a.r.	14	11	75	30
+ 9 h a.r.	26	6	68	15
Log-culture	45	43	12	5

the G_2 phase. For this purpose A431 cells were grown in plastic dishes to a density of 10^5 cells/cm² and then treated with 1×10^{-6} M amethopterin and 5×10^{-5} M adenosine for 16 h in complete medium; the blockage was then released by the addition of thymidine ($10 \mu\text{g}/10^6$ cells; [21]). For the demonstration of synchrony flow-cytometric measurements of DNA distribution of the cultures were taken in parallel as described [21]. Using this kind of analysis G_2 cells and mitotic cells can not be separated. Therefore in parallel a time-lapse photography experiment was performed starting 7 h after release in order to evaluate the total number of mitoses from the pictures. By this calculation 7.5–8 h after release about 50% of the cells were found to be in G_2 phase (Table 1). Treatment with PMA started 7.5–8 h after release from amethopterin blockage, i.e. at the time of maximum enrichment (about 6–7-fold) of cells in G_2 phase.

Qualitative analysis of phospholipid metabolites

For radioactive prelabelling of phospholipids 4×10^5 cells were transferred into plastic Petri dishes (3.5-cm-diam.; Falcon). After 24 h the exponentially growing cultures received [$1-^{14}C$]oleic acid or [$1-^{14}C$]arachidonic acid (each 7.4 kBq/ml) dissolved in DMEM plus 10% (v/v) calf serum for 24 h. The medium was then replaced by DMEM containing 0.5% BSA. After 2 h of equilibration the cells were pretreated for 10 min with the alcohols (0.3%) or PBS as control; they were then incubated with PMA or acetone (0.2%).

In synchronized cell cultures the cells were prelabelled for 22 h with [$1-^{14}C$]oleic acid during the incubation period with amethopterin and treated with PMA as described above.

Extraction and separation of lipids

The incubation of cells with agonists was stopped at the indicated time points by removing the medium and adding 2 ml of ice-cold methanol to the cells. The extraction of lipids was performed with chloroform/methanol (1:1, v/v) and 1 mM EGTA in 1 M HCl [22]. Separation of phosphatidic acid and phosphatidylpropanol was performed on Silicagel G 60 TLC plates using the upper phase of ethylacetate/iso-octane/acetic acid/water (130:20:30:100, by vol.) as a solvent system with dioleoyl phosphatidic acid and phosphatidylpropanol as standards. The latter was prepared as described below. Separation of neutral lipids was performed in a solvent system consisting of benzene/chloroform/methanol (80:15:5, by vol.). Radioactivity was measured with a Linear Analyser (Berthold, Wildbad, Germany).

Figure 1 Effect of PMA on the G₂-M transition of A431 cells

A431 cells were cultivated for 48 h, and after medium change with DMEM containing 0.5% BSA and 2 h of equilibration they were treated at zero time with acetone (0.2%; ○) or PMA (1×10^{-7} M; ●) for the periods indicated. The entry of cells into mitosis was monitored by time-lapse photography as described in the Materials and methods section. (A) Number of cells entering prophase in 10 min intervals after PMA treatment (individual experiments). (B) Cumulative number of cells entering prophase. Each curve represents the sum of two experiments.

Table 2 Quantitative analysis of the PMA-induced formation of phosphatidic acid

Cells were cultivated for 48 h, and 2 h after medium change with DMEM containing 0.5% BSA they were incubated for 30 min with acetone (0.2%) or PMA (1×10^{-7} M). Quantification of phosphatidic acid was performed by Coomassie Blue staining as described in the Materials and methods section.

Treatment (30 min)	Phosphatidic acid formation ($\mu\text{g}/10^6$ cells)	Diacylglycerol formation ($\mu\text{g}/10^6$ cells)
Acetone	0.1 ± 0.027	0.3 ± 0.017
PMA	0.205 ± 0.012	0.301 ± 0.001

Preparation of the phosphatidylpropanol standard

For the preparation of the phosphatidylpropanol standard, phospholipase D from *Streptomyces* sp. (Sigma Type VII) was used. The reaction mixture contained 80 mM sodium acetate, pH 5.6, 28% (v/v) diethyl ether, 1 mg of L- α -dioleoyl phosphatidylcholine, 5% propanol and 0.2 unit of bacterial phospholipase D in a total volume of 500 μl . After stirring for 30 min at 37 °C the reaction was stopped by adding 200 μl of 6 M HCl. Lipids were extracted with 800 μl of chloroform/methanol (1:1, v/v). Separation of phosphatidylalcohols was performed on TLC plates as described above. The spots were visualized using iodine vapour. For purification of phosphatidylpropanol spots were scraped off the TLC plates and dissolved in 2 ml of chloroform/methanol (1:1, v/v). After filtration of the silica gel and evaporation of the solvent the lipids were dissolved in 500 μl of the chloroform/methanol mixture and 10 μl aliquots were used as standards. A standard curve with phosphatidylpropanol was obtained by phosphorus determination [23]. Evaluation of

the amount of cellular phosphatidylpropanol was performed by using the molecular mass of dioleoyl phosphatidylpropanol (743 g/mol) as the basis.

Quantitative analysis of phospholipid metabolites

For determination of the total amounts of diacylglycerol 4.5×10^5 cells were cultivated in 3.5 cm-diam. dishes, for that of phosphatidic acid and phosphatidylpropanol 1.3×10^6 cells were cultivated in 5-cm-diam. dishes for 48 h. After a medium change with DMEM + 0.5% BSA and equilibration for 2 h, cells were treated with the substances to be tested. The extraction and separation of the lipid metabolites were performed as described above using 1,3-dioleoylglycerol or 1,2-dioleoyl phosphatidic acid and phosphatidylpropanol as standards over the linear range (0.4–1.6 μg). After developing the lipids in the solvent systems described above the TLC plates were stained for 30 min in a solution of 0.03% Coomassie Brilliant Blue R-250 (Serva, Heidelberg, Germany) in 30% methanol/100 mM NaCl and destained for 5 min with 30% methanol/100 mM NaCl according to [24]. The density of each spot co-migrating with the standards was measured with a Video-densitometer Bio-Profil (Vilber-Lourmat/Fröbel, Lindau, Germany).

RESULTS

PMA-induced inhibition of the G₂/M transition in A431 cells

Treatment of exponentially growing A431 cells with PMA (1×10^{-7} M) 2 h after the change to DMEM/BSA led to a rapid decrease in the number of cells entering prophase (Figure 1A), i.e. an inhibition of the transition from G₂ phase to mitosis. Cells which were already engaged in mitosis at the beginning of the PMA treatment continued division. Later on cells recovered

Figure 2 Time course of the PMA-induced formation of phosphatidic acid (A,B) and phosphatidylpropanol (C,D)

(A and C) A431 cells were cultivated for 48 h and then received a medium change with DMEM containing 0.5% BSA. After 2 h cells were pretreated for 10 min with 1-propanol (0.3%; ■) and then incubated with acetone (0.2%; ○) or PMA (1×10^{-7} M; closed symbols) for the periods indicated. The quantification of phospholipids was performed by Coomassie Blue staining and densitometry as described in the Materials and methods section. (B and D) After prelabelling for 24 h with $[1-^{14}\text{C}]$ oleic acid, medium change with DMEM containing 0.5% BSA and 2 h of equilibration the cells were treated as described in (A) and (C). The analysis of radioactive phospholipids was performed with a Linear Analyser. Each value represents the mean $\pm$ S.D. of three cultures.

from the inhibition and resumed the transition to prophase. The delay time was about 90 min compared with the control treated with 0.2% acetone, as indicated by the plot of the cumulative number of cells entering prophase (Figure 1B) (determined at the level $\log(N/N_0) \times 10^2 = 1$). At 3×10^{-8} M, PMA was active to a similar degree as at a concentration of 1×10^{-7} M. At 1×10^{-8} M PMA caused a delay of about 40–50 min (results not shown).

PMA-induced generation of phospholipid metabolites in A431 cells

This rapid cellular response and recovery from inhibition in the presence of the inducing ligand PMA is consistent with the idea of a mediator responsible for the transient arrest of A431 cells in G_2 phase. Measurements of potential candidates [8] in A431 cells in response to PMA showed an increase of phosphatidic acid but not of diacylglycerol (Table 2) under these conditions. From repeated experiments it became evident that the amount of phosphatidic acid was approximately doubled within a short period of time.

The time course of phosphatidic acid formation in A431 cells

treated with PMA after the change of medium is shown in Figures 2(A) and 2(B). It mirrors the time course of the G_2 inhibition. A maximum phosphatidic acid level is seen after 10 min. After about 60 min the increased phosphatidic acid level began to return to the control level, almost reaching this level after about 90 min (Figure 2A). The generation of radioactively labelled phosphatidic acid in response to PMA treatment was only observed in A431 cells prelabelled with radioactive oleic acid (Figure 2B) and was not seen in cells prelabelled with radioactive arachidonic acid (see below). The time course of the generation of radiolabelled phosphatidic acid in cells prelabelled with oleic acid basically reflected that of the absolute amounts formed (cf. Figures 2A and 2B). The cell-cycle data, as well as the biochemical data, correlated strongly with respect to the onset of the reaction and its duration. An overnight pretreatment of A431 cells with PMA (1×10^{-7} M) completely abolished the responsiveness to a second PMA treatment with respect to both cell-cycle interference and phosphatidic acid formation (results not shown).

In A431 cells labelled with oleic acid radioactivity is pre-

Figure 3 Dose-response relationships for the formation of phosphatidic acid (A) and phosphatidylpropanol (B) in response to PMA

Cells were prelabelled for 24 h with [¹⁴C]oleic acid. Two hours after medium change with DMEM + 0.5% BSA they were pretreated for 10 min with 1-propanol (0.3%; ■) or PBS (●) and then incubated for 30 min with the PMA concentrations indicated. Each value represents the mean $\pm$ S.D. of three cultures.

Figure 4 Effect of 1-propanol (A) and 2-propanol (B) on the PMA-induced G₂ delay of A431 cells

Cells were pretreated for 10 min with 1-propanol (□, ■), 2-propanol (△, ▲; each 0.3%) or PBS (○, ●) and incubated at zero time with acetone (0.3%; ○, □, △) or PMA (1 × 10⁻⁷ M; ●, ■, ▲). The entry of cells into mitosis was monitored by time-lapse photography in 10 min intervals. Each curve represents the sum of two experiments.

dominantly found in phosphatidylcholine, whereas preincubation of cells with radiolabelled arachidonic acid led to a prominent labelling of phosphatidylserine and phosphatidylinositol [8]. The total lack of arachidonyl phosphatidic acid after PMA treatment seems to point to a strict separation of phos-

pholipid pools (i.e. of phospholipid species carrying different fatty acid moieties), and/or to a relatively strict substrate specificity of the responsible phospholipase. The results may indicate that phosphatidic acid mobilized in response to PMA originates largely from phosphatidylcholine. This idea is sup-

Figure 5 Influence of 1-propanol on the EGF-induced formation of phosphatidic acid (A) and the EGF-induced G_2 delay (B)

(A) Cells were pretreated for 10 min without or with 1-propanol (1-OH; 0.3%) and then incubated for 30 min with EGF (1×10^{-7} M) or PBS. The quantitative analysis of phosphatidic acid was performed as described in the Materials and methods section. Each bar represents the mean $\pm$ S.D. of three cultures. (B) Cells were pretreated for 10 min with 1-propanol (0.3%; $\square$, $\blacksquare$) or PBS ($\circ$, $\bullet$). At zero time they were incubated with EGF (1×10^{-7} M; $\bullet$, $\blacksquare$) or PBS ($\circ$, $\square$). The entry of cells into mitosis was monitored by time-lapse photography. Each curve represents the sum of two experiments.

Figure 6 Effect of 1- and 2-propanol on the PMA-induced formation of phosphatidic acid (A) and phosphatidylpropanol (B) in synchronized A431 cells at the G_2 -M border

Cells were synchronized as described in the Materials and methods section. In parallel to the synchronization procedure cells were prelabelled for 22 h with [^{14}C]oleic acid. The cells were pretreated, 8 h after release from the amethopterin block, for 10 min with 1-propanol (1-OH) or 2-propanol (2-OH), each at 0.3%, and then incubated for 30 min with PMA (1×10^{-7} M; $\blacksquare$) or acetone (0.2%; $\square$) without changing the medium. The analysis of phosphatidic acid (A) and phosphatidylpropanol (B) was carried out as described in the Materials and methods section. Each value represents the mean $\pm$ S.D. of three cultures.

ported by the specificity of the enzyme responsible, i.e. phospholipase D (see below), which seems to hydrolyse phosphatidylcholine preferentially [25].

PMA-induced pathway of phosphatidic acid formation and modulation by 1-propanol

The fact that after PMA treatment of A431 cells an increase in phosphatidic acid only could be detected, and not in diacyl-

glycerol, led to the assumption that most of the phosphatidic acid derived from activation of a phospholipase D. Such a reaction has already been shown in several cell lines [26–28]. A characteristic of this enzyme is its ability to transfer phosphatidate from phospholipids preferentially to primary alcohols [17–20]. In this case phosphatidylalcohols are formed at the expense of phosphatidic acid. For the purpose of this cell-cycle study it was important, first, that the transphosphatidylation reaction takes place in intact cells and, secondly, that a significant shift towards

Figure 7 Effect of 1- and 2-propanol on the PMA-induced G₂ delay in synchronized A431 cells

Following release (after 8 h) from the amethopterin block cells were pretreated for 10 min with PBS (A), 1-propanol (B) or 2-propanol (C; each at 0.3%), and were then incubated at zero time with acetone (0.2%; ○, □, △) or PMA (1×10^{-7} M; ●, ■, ▲). The entry of cells into mitosis was monitored by time-lapse photography in 3 min intervals. Each set of experiments was performed at the same day with parallel cultures under comparable conditions and was repeated with similar results.

the formation of phosphatidylalcohol occurs at low alcohol concentrations which do not interfere to any great extent with the cell-cycle progress (see below). In order to test the role of phospholipase D in PMA-treated A431 cells, cultures were incubated with 1-propanol (0.3% final concentration) 10 min prior to the addition of PMA or acetone (0.2% final concentration) for control reactions (0.3% alcohol does not itself result in activation of phospholipase D in A431 cells [29]). The data obtained with a maximally active dose of PMA (1×10^{-7} M) (Figures 2A and 2B) demonstrate that in the presence of the primary alcohol the degree of phosphatidic acid formation is reduced almost to the level of the control. Instead of phosphatidic acid, the cells form phosphatidylpropanol (Figures 2C and 2D). This process is also evident when using different PMA concentrations (Figures 3A and 3B). The increase of phosphatidylpropanol formed in A431 cells in response to PMA far exceeded that of phosphatidic acid induced in the absence of the primary alcohol (about 0.17 μ g of phosphatidylpropanol at 30 min versus 0.07 μ g of phosphatidic acid at this time). This result may be explained by the relative metabolic stability of the phosphatidylalcohol.

The use of 1-propanol in this study allowed application of the isomer 2-propanol as a control, a secondary alcohol which should not be accepted by phospholipase D as a substrate. Unlike 1-propanol, 2-propanol did not reduce PMA-induced phosphatidic acid formation and did not result in the formation of phosphatidylpropanol (see below).

Modulation of PMA-induced G₂ delay by propanol

Taken together the results shown in the previous section indicate that, under the conditions used, the PMA-induced formation of phosphatidic acid was largely effected through phospholipase D and that it could be greatly reduced in the presence of 1-propanol but not 2-propanol. With this background it became possible to employ the transphosphatidylation reaction for the reduction of

phosphatidic acid formed in response to PMA and to measure the consequences for the G₂/M transition. On the basis of the biochemical data a set of experiments was carried out using 1×10^{-7} M PMA and a final concentration of 0.3% 1-propanol. The results are summarized in Figure 4(A) where they are plotted as cumulative number of cells entering prophase. They show that 1-propanol reduced the PMA-induced G₂ delay from 90 min to approx. 30 min [at the level $\log(N/N_0) \times 10^2 = 1$]. In contrast, the secondary alcohol 2-propanol, which did not influence PMA-induced phosphatidic acid formation, was unable to interfere with the onset and the duration of the G₂ delay induced by PMA compared with the relevant controls (Figure 4B). The acceleration of the recovery from the delay may be due to unspecific alcohol effects. The efficacy of 1-propanol is particularly surprising in view of the relatively large amounts of phosphatidylpropanol formed at the same time. These results suggest that phosphatidic acid has a biological potency which differs widely from that of the corresponding phosphatidylalcohol.

Influence of 1-propanol on the EGF-induced G₂ delay

A further possibility for highlighting the role of phosphatidic acid in ligand-induced cell-cycle delay is to test the effect of 1-propanol in a system where only a relatively small reduction of the amount of phosphatidic acid caused by the alcohol treatment could be observed, namely by treating A431 cells with EGF [29]. We have reported that after incubation of cells with EGF most of the phosphatidic acid formed derived from the phospholipase C/diacylglycerol kinase pathway. Therefore it was expected that the G₂ delay induced by EGF (1×10^{-7} M) would not be influenced by a primary alcohol. The result in Figure 5 shows that under conditions where the EGF-induced increase in phosphatidic acid was only partially reduced (Figure 5A) the alcohol had no effect on the extent of the EGF-induced G₂ delay (Figure 5B). This indicates that only a significant reduction of phosphatidic acid level leads to a reduction of the ligand-induced G₂

Figure 8 Effect of exogenous phospholipase D on the formation of phosphatidic acid (A) and the G_2 -M transition of synchronized A431 cells (B)

(A) Following release (after 8 h) from the amethopterin block cells were treated for 30 min with phospholipase D from *Streptomyces chromofuscus* (2 units/ml; PLD), PMA (1×10^{-7} M) or PBS. The amount of phosphatidic acid was calculated as $\mu\text{g}/\text{nmol}$ of phospholipid phosphorus as described in the Materials and methods section. Each value represents the mean $\pm$ S.D. of three cultures. (B) Following release (after 8 h) from the amethopterin block cells were treated with phospholipase D (2 units/ml; $\bullet$) or PBS ($\circ$). The entry of cells into mitosis was monitored by time-lapse photography in 3 min intervals. Each experiment was performed twice with similar results.

delay and strongly supports the role of phosphatidic acid as a potential mediator.

PMA-induced activation of phospholipase D in synchronized A431 cells

The cell-cycle studies described up to this point were performed by a single-cell analysis of cells entering mitosis, whereas the biochemical results reflect the reactions of entire logarithmically growing cultures, which contain about 5–10% of cells in G_2 phase/mitosis (Table 1). In order to measure the influence of PMA on the formation of lipid mediators in G_2 cells, A431 cultures were synchronized [21]. For this purpose cells were predominantly inhibited at the G_1/S border (about 70%; Table 1) by amethopterin treatment for 17 h. Then they were released from the block by thymidine and allowed to continue in the cell cycle to the G_2 phase, which was reached by the majority of cells after about 8 h. Under these conditions the PMA-induced activation of a phospholipase D was tested. The results in Figure 6(A) show that PMA activates a phospholipase D leading to an increase in phosphatidic acid. In the presence of 1-propanol an increase in phosphatidylpropanol could be observed, whereas the secondary alcohol was without effect (Figure 6B). This indicates (i) that the synchronization procedure did not interfere with the action of PMA, and (ii) that in contrast to the logarithmically growing A431 culture under these conditions a medium change was not necessary to visualize the metabolic response to the PMA treatment. The effect of PMA was investigated under these conditions on the G_2/M transition in the absence and presence of 1- and 2-propanol. Cells were pretreated, 7 h after release, with 1- or 2-propanol, or PBS as control, and then incubated with PMA (1×10^{-7} M) or acetone (at time zero). In order to measure the mitotic activity with a smaller window at the G_2/M border an interval of 3 min was chosen for the time-lapse photography.

The results in Figure 7(A) show that during the first 30 min many more cells in the control culture enter prophase than

compared with the control group in a logarithmically growing culture (Figure 2B), indicating a relatively high synchrony. After PMA treatment a clear reduction of the number of prophases with a rapid onset during the first 10 min could be observed, with a delay time of about 25 min (average of three different experiments; Figure 7A). In the presence of 1-propanol the PMA-induced delay was diminished almost to the control level (Figure 7B) but not in the presence of 2-propanol (Figure 7C). After about 30 min incubation the alcohols strongly accelerated the G_2/M transition independent of PMA treatment, indicating an unspecific stimulatory alcohol effect as seen also with asynchronous cells (see above). The results from the synchronization experiments support the data obtained with logarithmically growing cells and point to a role for phosphatidic acid as an inhibitory mediator of the PMA-induced G_2 delay.

Influence of an exogenous phospholipase D on the lipid metabolism and the G_2/M transition in synchronized cells

In order to evaluate the role of phosphatidic acid in this respect from a different point of view an exogenous bacterial phospholipase D from *Streptomyces chromofuscus* was used to mobilize phosphatidic acid directly in the G_2 population of synchronized A431 cells. A concentration of phospholipase D (2 units/ml) was used which mobilized significant amounts of phosphatidic acid comparable with the amounts elicited by PMA (calculated as μg of phosphatidic acid/nmol of phospholipid phosphorus) (see Figure 8A). (The formation of other lipid metabolites such as diacylglycerol, inositolphosphates or released arachidonic acid could not be observed; results not shown). The effect of this enzyme on the G_2/M transition was monitored by time-lapse photography. The results in Figure 8(B) show that very soon after phospholipase D treatment the cells were delayed for about 30 min from entering mitosis; this is comparable with the PMA-induced G_2 delay.

The results obtained in logarithmically growing as well as in synchronized A431 cells together strongly indicate the role of

phosphatidic acid as a potential mediator in the signal cascade activated by exogenous factors, which leads to a delay of the G₂/M transition of proliferating cells.

DISCUSSION

Previous studies in replicating cultured cells, including A431 cells, have shown that EGF, via its intact receptor, is able to cause a transient inhibition of cells in G₂ phase [4,5]. A search for inhibitory mediator(s) revealed phosphatidic acid as a possible candidate [7,8]. Since phosphatidic acid under these circumstances was substantially formed via phosphatidylinositol-specific phospholipase C and diacylglycerol kinase, a role of inositol phosphates in the G₂ delay could not be completely excluded.

By using PMA stimulation of phospholipase D [25–28], we could look at this pathway in the absence of any phospholipase C/diacylglycerol kinase contribution. The use of 1-propanol and 2-propanol in this system serves a dual purpose; it serves as a control to show that the phosphatidic acid is indeed being formed by the phospholipase D pathway (since only phospholipase D will transphosphatidate in the presence of primary but not secondary alcohols at the expense of phosphatidic acid), and in view of the lack of specific inhibitors for phospholipase D it serves as a means of shutting down phosphatidic acid production specifically, to demonstrate its immediate involvement in the G₂ delay.

One of the main effects in the presence of 1-propanol was to shift the balance of product from a negatively charged phosphatidic acid, capable of binding Ca²⁺ and Mg²⁺, to a lipid species which exhibits a different charge and probably a different location in cellular membranes. Omodeo-Salé et al. [30] reported that phosphatidylalcohols seem to exist in microdomains as 'clusters' and are relatively 'inert' to the attack of degrading enzymes reflected by their relatively high stability. In our studies this phosphatidylalcohol appears to be biologically inactive with respect to the G₂/M transition. Thus, the important point in our observations with the primary alcohol is the reduction of the amounts of phosphatidic acid elicited by the PMA treatment. Only under these conditions could a reduction of the G₂ delay be measured. In cases where the phosphatidic acid level could not be lowered by the presence of alcohols (PMA plus 2-propanol and EGF plus 1-propanol) the ligands predominated in the induction of a G₂ delay.

On the basis of earlier results, as well as of the data presented in this study, we tend to interpret the observations as indicative of an inhibitory role of newly generated phosphatidic acid on the PMA-induced G₂ delay in A431 cells.

A role of phosphatidic acid (as well as of lysophosphatidic acid) has already been implicated in growth control. Phosphatidic acid has been reported to be stimulatory for resting cells (for overviews see [9–13,31]). Agonist-induced activation of phospholipase D or application of exogenous phospholipase D have been shown to release cells from starvation arrest in G₀ phase and to cause them to enter another round of replication about 15 h later. Even though the time elapsing between activation of phospholipase D and the entrance into S phase appears to be rather long for connecting both events conclusively, an interesting target of phosphatidic acid has been discovered which may be related to its growth stimulatory activity. Phosphatidic acid has been shown to inhibit the activity of GAP, a GTPase-activating protein [32], as well as to stimulate the activity of a GAP-inhibiting protein [33]. Since both are directed towards the Ras protein both activities of phosphatidic acid are well suited to

result in an enhanced activity of Ras and Ras-related proteins and thus subsequently to contribute to the growth stimulus.

The situation in replicating cells is entirely different. Here the activation of phospholipase D and the increase in the level of phosphatidic acid correlate with a rapid inhibition in G₂ phase as we have shown. Since the proposed role of phosphatidic acid as an inhibitory mediator of the PMA-induced G₂ inhibition is supported by this study, the question arises as to what the possible function of phosphatidic acid might be.

The PMA treatment did not lead to a measurable increase in diacylglycerol. However, the use of propranolol, an inhibitor of the diacylglycerol-forming phosphatidic acid phosphohydrolase, resulted in an accumulation of phosphatidic acid (M. Kaszkin and V. Kinzel, unpublished work), indicating that phosphatidic acid was to some extent metabolized to diacylglycerol, which itself may undergo a rapid turnover. Furthermore, phosphatidic acid might be degraded to lipids other than diacylglycerol such as lysophosphatidic acid via a specific phospholipase A₂ [34]. Therefore, phosphatidic acid may act not just as a precursor for diacylglycerol but may exert additional functions. The rate of the formation of phosphatidic acid by the action of a phospholipase D exceeded its rate of degradation for a period of at least 1 h as we have measured in pulse experiments with 1-propanol (M. Kaszkin and V. Kinzel, unpublished work). Phosphatidic acid is known to exert biophysical effects in model membranes, which lead to the formation of non-bilayer structures and support fusion of vesicles with membranes [35,36]. However, there was no direct evidence for such an action of phosphatidic acid in cellular membranes. Recent findings concerning vesicle transport and membrane fusion seem to confirm descriptions of the properties of phosphatidic acid known since the 1970s.

It has been found by several groups, that the ADP-ribosylation factor (ARF), a small G-protein supporting the transport of coated vesicles to the plasmamembrane [37,38], also acts as a cytosolic cofactor for the activation of membrane-bound phospholipase D. In addition the presence of another negatively charged lipid, phosphatidylinositol 4,5-bisphosphate (PIP₂), seems to be important for this process [39]. There is increasing evidence that phosphoinositides play a non-signalling role in vesicular traffic in yeast and mammalian cells (for a review see ref. [40]). PIP₂ included in liposomes greatly enhances the activation of granulocyte phospholipase D by ARF. Phosphatidic acid in co-operation with PIP₂ is proposed to support the fusion processes between vesicles and the acceptor membrane because of their ability to form non-bilayer structures by changing the charge of the membrane surface. This phenomenon implies a role for phospholipase D and its product phosphatidic acid in vesicular trafficking. The PIP₂-producing enzyme phosphatidylinositol 4-phosphate 5-kinase (PIP-kinase) is present in secretory granules and coated vesicles from different cell types and can be activated by phosphatidic acid [41]. The connection between the activation of the PIP-kinase by phosphatidic acid and the subsequent positive feedback activation of phospholipase D by PIP₂ in concert with ARF was illustrated in a model by Liscovitch et al. [39].

How do the ligand-induced G₂ delay and the parallel formation of phosphatidic acid fit into this model? Vesicular transport- and fusion-processes are normally inhibited when cells enter mitosis, as was shown by Warren [42,43]. The Golgi complex, which exists in a single copy in the cell, reaches a vesiculated state in order to be partitioned equally between the two daughter cells. In the case of an increase in phosphatidic acid the vesicle budding and fusion supported by ARF-GTP continue until the feedback loop is interrupted by degradation of the amounts of lipid to permissive values or by shutting down of the cycle by phos-

phatidic acid itself. The idea is that phosphatidic acid activates the ARF-activated GTPase leading to the inactive form ARF-GDP [44]. As long as enough phosphatidic acid is present and this process proceeds, the cells are not able to prepare for entry into mitosis. In the presence of a primary alcohol there may not be enough phosphatidic acid formed to activate the PIP-kinase and the ensuing cascade, and phosphatidylalcohols seem to be inactive in this process. Under these conditions the vesiculation of the membranes is no longer disturbed and the cells start their preparation for mitosis. Thus the initial increase in significant amounts of phosphatidic acid directly at the G_2/M border seems to be of prime importance. The sensitivity of cells to an increase in phosphatidic acid at this point in the cell cycle is indicated in particular by the experiments with exogenous ligands as well as with the exogenous phospholipase D in synchronized cells. These investigations gave a clear picture of their inhibitory effects on the G_2/M transition and the corresponding increase in phosphatidic acid.

The major component of intracellular growth control at the G_2/M transition downstream of the ligand binding and activation of phospholipases is the mitosis-promoting factor (MPF). It consists of a protein kinase p34^{cdc2} in complex with cyclin B [45–47]. Activation of the enzyme is absolutely required for the transition from G_2 phase to mitosis, an event which is achieved by dephosphorylation of p34^{cdc2} at specific residues [48–50]. This would suggest that the increase in phosphatidic acid level may be part of the cascade which prevents activation of the p34^{cdc2}-protein kinase activity and thus prevents the transit from G_2 phase to mitosis. It was reported by Barth and Kinzel [51,52], that EGF as well as PMA prevented the activation of the p34^{cdc2}-kinase in HeLa cells. Furthermore it could be shown by Barth et al. (H. Barth, I. Hoffmann, S. Klein, M. Kaszkin, J. Richards and V. Kinzel, unpublished work), that both ligands seem to inactivate the cdc25-C-phosphatase in HeLa cells, which is responsible for the activation of the kinase. Preliminary experiments carried out with A431 cells have provided comparable results (M. Kaszkin, H. Barth and V. Kinzel, unpublished work).

The arrest of the vesiculation processes at the G_2/M border seems to be connected with the action of the p34^{cdc2}-kinase, as was shown *in vitro* by Tuomikoski et al. [53]. It is tempting to speculate that the increase in phosphatidic acid and the ARF-promoted transport and fusion of vesicles act against the activation of the p34^{cdc2}-kinase, leading to a delay in the cell-cycle progression at the G_2/M border.

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