Arachidonic acid mobilization is suppressed during mitosis: role of cytosolic phospholipase A₂ activation

Richard D. BERLIN* and Susan F. PRESTON

Department of Physiology, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT, U.S.A.

In interphase HeLa cells, incubation with histamine or thapsigargin led to the rapid release of arachidonic acid. The release was absolutely dependent on Ca²⁺, consistent with the activation of an 85 kDa cytosolic phospholipase A₂ (cPLA₂). In metaphase-arrested HeLa cells, by contrast, the stimulation of arachidonate release by these agents was inhibited by more than 90%. The lack of arachidonic acid release by mitotic cells was at least partly expected, since histamine- or thapsigargin-induced Ca²⁺ influx and elevations of cytosolic free Ca²⁺ are known to be strongly inhibited during mitosis [Preston, Sha'afi and Berlin (1991) *Cell Regul.* 2, 915–925]. Indeed, incubation of interphase cells with the Ca²⁺ ionophore A23187 alone induced a high level of arachidonate release. However, even A23187 failed to elicit release from mitotic cells. Since the Ca²⁺-dependent release of arachidonate by many cell types is promoted by preincubation with ligands that activate receptors of the tyrosine kinase class, and tumour promoters that lead to the phosphorylation of

cPLA₂, we determined if the responses of mitotic HeLa cells could be modified by this 'priming' process. We first established that epidermal growth factor and phorbol 12-myristate 13-acetate were effective priming agents in interphase cells: cells preincubated with the hormone or tumour promoter showed a 2-fold stimulation of thapsigargin- or A23187-induced arachidonic acid release. However, none of the priming agents reversed the lack of mitotic cell response. This refractoriness was not caused by destruction of cPLA₂ during mitosis: by Western blotting, cPLA₂ of interphase and mitotic cells was shown to be present in comparable amounts. Moreover, cPLA₂ activities measured in extracts of interphase and mitotic cells were also comparable. Surprisingly, mitotic cPLA₂ appeared to be constitutively phosphorylated in non-hormone-treated (control) cells. The results indicate a novel mechanism of regulation of cPLA₂ activity in mitotic cells.

INTRODUCTION

Mitotic cells are profoundly altered in their membrane functions: pinocytosis [1], exocytosis [2,3], receptor recycling [4,5] and Ca²⁺ influx induced by hormones [6,7] are strongly inhibited. We have studied these changes to suggest new ways in which membrane functions can be regulated [6,8]. In this study, we show that the mobilization of arachidonate in response to hormones is strongly inhibited in mitotic cells. This places significant constraints on the physiological consequences of hormone stimulation in mitotic cells and, at the same time, presents a natural model in which the regulation of arachidonate mobilization can be examined.

The production of arachidonic acid is generally held to be the rate-limiting step in the formation of eicosanoids such as the prostaglandins, and is essential for the actions of cytokines and diverse hormones (e.g. [9–12]). The release of arachidonic acid from phospholipid depends primarily on the activation of an arachidonic acid-selective cytosolic phospholipase A₂ (cPLA₂). It has recently become clear from studies with the purified selective cPLA₂ that maximal activation is dependent on the synergistic actions of at least two factors: an elevation of intracellular free calcium ([Ca²⁺]_i), which is essential for catalysis and appears to promote the translocation of the cytosolic enzyme to its membrane substrate [13,14], and specific phosphorylation of the enzyme [10,15], probably by MAP (mitogen-activated protein) kinase [16]. However, there are other sites of phosphorylation [15]. Moreover, it is unclear if phosphorylation and free Ca²⁺ levels can account for the entire regulation of cPLA₂ *in vivo*.

It is reasonable to expect that Ca²⁺-dependent arachidonate

mobilization will be affected during mitosis. The hormone-induced elevation of cytosolic free Ca²⁺ is not sustained by mitotic cells [6,7]. Whereas during interphase depletion of intracellular Ca²⁺ stores induces Ca²⁺ influx (capacitive coupling [17]), during mitosis, depletion of Ca²⁺ stores does not induce Ca²⁺ influx [6]. The Ca²⁺-dependent translocation of cPLA₂ to the membrane, and catalysis, are therefore likely to be inhibited in mitotic cells. In addition, several protein kinases, some with broad substrate specificity, are specifically activated during mitosis [18,19], raising the possibility that the phosphorylation-dependent regulation of cPLA₂ might also be altered in mitotic cells.

We show here that Ca²⁺-dependent arachidonate mobilization is, indeed, strongly inhibited in metaphase-arrested HeLa cells. More surprisingly, although elevation of cytosolic Ca²⁺ by addition of the Ca²⁺ ionophore A23187 leads to substantial arachidonate release from interphase cells, addition of A23187 to mitotic cells does not induce release. However, quantitative Western blots show that the amount of cPLA₂ is comparable in interphase and mitotic cells. Furthermore, the mitotic cPLA₂ appears to be constitutively phosphorylated and is active *in vitro* in a Ca²⁺-dependent manner.

EXPERIMENTAL

Materials

[5,6,8,9,11,12,14,15-³H]Arachidonic acid (100 Ci/mmol) was purchased from Dupont-NEN (Boston, MA, U.S.A.). 1-Stearoyl-2-[¹⁴C]arachidonyl phosphatidylcholine (55 Ci/mmol)

Abbreviations used: PLA₂, phospholipase A₂; cPLA₂, cytosolic PLA₂; [Ca²⁺]_i, intracellular free Ca²⁺ concentration; EGF, epidermal growth factor; TNF-α, tumour necrosis factor-α; PMA, phorbol 12-myristate 13-acetate; PAP, potato acid phosphatase; BAPTA, 1,2-bis-(2-aminophenoxyethane)-NNN'-tetra-acetic acid; BAPTA-AM, BAPTA acetomethoxy form; DTT, dithiothreitol; FBS, fetal-bovine serum; MAP kinase, mitogen-activated protein kinase.

* To whom correspondence should be addressed.

and ECL reagents were from Amersham (Arlington Heights, IL, U.S.A.). Non-labelled 1-stearoyl-2-arachidonyl phosphatidylcholine was obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Murine monoclonal anti-cPLA₂ antibody was generously provided by Dr. J. L. Knopf, Genetics Institute (Cambridge, MA, U.S.A.). Immobilon polyvinylidene difluoride membrane was obtained from Millipore (Bedford, MA, U.S.A.). Tissue-culture media and fetal-bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, U.S.A.). Recombinant human epidermal growth factor (EGF) was obtained from Chiron (Emeryville, CA, U.S.A.). 1,2-Bis-(2-amino-phenoxyethane)-*NNN'*-tetra-acetic acid, acetomethoxy form (BAPTA-AM), and 4-bromo A23187 were purchased from Molecular Probes (Eugene, OR, U.S.A.); thapsigargin was from LC Services Corp. (Woburn, MA, U.S.A.).

Hoechst 33258 was purchased from Calbiochem (La Jolla, CA, U.S.A.). XAR film was from Kodak. Protein concentrations were determined by using the Bio-Rad protein reagent from Bio-Rad (Richmond, CA, U.S.A.). LK6D and silica-gel 60 TLC plates were obtained from Whatman (Clifton, NY, U.S.A.). All other chemicals and reagents, including potato acid phosphatase (PAP; type III), phenylmethanesulphonyl fluoride, leupeptin, aprotinin, mepacrine, Ca²⁺ ionophore A23187, fatty-acid-free BSA, histamine, dithiothreitol (DTT), phorbol 12-myristate 13-acetate (PMA), colcemid, arachidonic acid and 1,2-diacylglycerol were purchased from Sigma (St. Louis, MO, U.S.A.).

Preparation of metaphase-arrested and interphase cell suspensions

HeLa-S3 cells were seeded at 10⁵ cells/ml on 100 mm Falcon tissue-culture dishes and grown in monolayer in F-12 medium (Ham's nutrient mix) supplemented with L-glutamine and 7.5 % FBS as previously described [6,7].

The following procedures resulted in minimal contamination of mitotic populations with interphase cells [6]. Mitotic cells were obtained after the addition of colcemid (0.1 µg/ml) to monolayers for 15 h, followed by detachment of the rounded-up cells by gentle shaking. This simple method provided nearly pure populations of mitotic cells (> 95 %, as determined by staining with Hoechst dye 33258 [4]). To control for long drug exposure, in selected experiments (representative of each protocol described below), cells were first synchronized by single thymidine block, then collected by shake-off ~ 16 h after removal of thymidine into colcemid, and held for only 1–5 h. Allowing for the contribution of interphase cells to the mitotic population of cells obtained with thymidine block, there was no difference in the behaviour of mitotic cells collected by the two methods. In addition, incubation of interphase cells in colcemid for 5–6 h did not affect their responses.

Unlike Wheatley et al. [20], who grew cells in somewhat different media, we found that ~ 80 % of cells collected even after 15 h in colcemid attached to tissue-culture plastic and progressed into G1 upon removal of the drug. Their long-term behaviour was not analysed.

To create interphase populations similar to mitotic cells that were detached from substrate, we transferred cells from Falcon tissue-culture plastic to Petri dishes, on which growth occurs essentially in suspension. Under our culture conditions, HeLa cells grown for 24 h on Petri dishes remain rounded and are easily detached without trypsin.

Release of [³H]arachidonic acid from intact cells

After initial plating, cells were cultured for 24 h. Medium was

then removed and replaced with fresh F-12 medium containing 7.5 % FBS and 1.0 µCi/ml [³H]arachidonic acid for up to 18–20 h. The time course of [³H]arachidonate uptake by interphase and mitotic cells and the resulting radioactivity (c.p.m./mg of cell protein) were essentially identical, with 70–80 % of label incorporated in 3 h (results not shown). Using the method of lipid analysis described below, we found that < 1 % of the cell-associated radioactivity was in non-esterified fatty acid. This was true for both mitotic and interphase populations, even in the presence of agents which stimulated arachidonate release. As shown by others [21], the label was found in all classes of phospholipid. Further, the time course of uptake was similar to that reported for HeLa cells by Espe et al. [21]. In general, it was advantageous to avoid an additional incubation after cell harvesting by allowing incorporation of label during the overnight period used to prepare metaphase-arrested cells. Comparable labelling periods were used for interphase cells. The labelling procedure did not affect the pattern of release.

Labelled interphase and mitotic populations were centrifuged at 600 *g* for 5 min, washed in fresh growth medium, and resuspended at 10⁶ cells/ml in F-12 with 7.5 % FBS buffered with 10 mM Na-Hepes, pH 7.4. To investigate the potential contribution of serum to fatty acid release, for selected experiments, cells were washed twice and resuspended in growth medium with decreased (0.5 %) serum, or in serum-free medium (buffered with 30 mM Na-Hepes) to which 1.0 mg/ml fatty-acid-free BSA was added [22]. (Albumin serves as an extracellular trap for released arachidonic acid). Under our conditions, the decrease or absence of serum did not significantly alter either the qualitative or quantitative pattern of agonist-stimulated [³H]arachidonic acid release. Therefore, unless specifically stated otherwise, arachidonic acid release data were gathered from samples with 7.5 % FBS at 37 °C present throughout.

Samples (10⁶ cells in 1.0 ml) in microfuge tubes were incubated at 37 °C with appropriate stimuli or inhibitors for times indicated in the Figure legends, then centrifuged at 14000 *g* for 5 s to separate cells from medium. Samples of medium were removed for immediate determination of radioactivity, and cell and medium samples were processed for lipid analysis [23].

Lipid analysis

Both medium (0.2 ml samples) and cells (pelleted cells washed twice in ice-cold serum-free F-12 buffered with 30 mM Na-Hepes and supplemented with 1.0 mg/ml fatty-acid-free BSA, and then resuspended in 0.2 ml of this medium) were added to glass tubes containing 1.0 ml of methanol. Samples were vortex-mixed and kept at room temperature for 1 h to extract lipids. The methanol fraction was removed, and the aqueous phase was re-extracted to give a final methanol extraction volume of 2 ml. The methanol fraction was vortex-mixed with 2 ml of chloroform, followed by 2 ml of 2 M KCl/5 mM EDTA. Chloroform and methanol layers were separated by centrifugation at 600 *g* for 10 min. The chloroform layer was dried down under N₂ and samples were resuspended in chloroform/methanol (2:1, v/v). Only background radioactivity remained in the aqueous phase after extraction.

Lipids were separated by TLC on silica-gel 60 in heptane/isopropyl ether/acetic acid (60:43:3, by vol.), revealed with I₂, and spots corresponding to arachidonic acid, diacylglycerol and phospholipid were scraped and counted for radioactivity in 10 ml of scintillation fluid in a Beckman LS 7500 scintillation counter.

Virtually all of the radioactivity released in these experiments,

regardless of the duration of uptake and labelling time, was associated with non-esterified fatty acid.

Preparation of cell extracts and measurement of cPLA₂ activity *in vitro*

With slight modification, extracts were prepared and cPLA₂ activity was measured as described previously [22,24]. At 24–48 h after plating, cells were washed and resuspended (10⁶ cells/ml) in serum-free F-12 medium buffered with 30 mM Na-Hepes, pH 7.4, containing 1.0 mg of fatty-acid-free BSA/ml. Samples were incubated in this medium for 2 h at 37 °C, after which stimulatory agents were added as indicated in the Results section and in Figure and Table legends and incubated for an additional 20 min. Cells were then washed three times by centrifugation in homogenization buffer (50 mM Hepes, pH 7.4, 250 mM sucrose, 1 mM EDTA and 1 mM EGTA), and the pellets resuspended in a small volume (200–400 µl) of homogenization buffer containing 2 mM phenylmethanesulphonyl fluoride, leupeptin and pepstatin (40 µg/ml each) and aprotinin (80 µg/ml). Samples were probe-sonicated on ice (two 20 s exposures to the tip of a Kontes micro ultrasonic cell disrupter, tune dial set on 5). The lysate was then centrifuged at 1000 *g* for 5 min to remove nuclei and debris. Cytosol samples were collected as supernatants obtained by centrifugation at 100 000 *g* for 1 h at 4 °C. The excess of Ca²⁺ chelators during preparation of the extracts is required to obtain maximal cPLA₂ activity in the supernatant fraction by dissociating the enzyme from membranes [13,22]. Protein in the extract was determined by the Bio-Rad assay [25].

Substrate was prepared by drying down 1-stearoyl-2-[¹⁴C]-arachidonyl phosphatidylcholine under N₂, and resuspending in dimethyl sulphoxide by vigorous vortex-mixing. Reaction mixtures (40 µl total volume) were formed as follows: cell-free extract (34 µl of extract adjusted with homogenization buffer to final concentrations in the range 0.3–1.0 mg/ml) was added to microfuge tubes containing 2 µl of substrate in dimethyl sulphoxide (~70 000 c.p.m./tube, final concn. 15 µM); the reaction was initiated by addition of 4 µl of 40 mM CaCl₂ in 30 mM Hepes, pH 7.5 (final concn. 4 mM Ca²⁺, 2 mM in excess of chelators). As reported [22], Ca²⁺ was absolutely required for cPLA₂ activity in the enzyme assay. DTT (5 mM) was present in either the extract or the reaction mixture. DTT destroys the secretory form of PLA₂ [24,26] and was used routinely in assays for cPLA₂. However, in fact the extract activity was unaffected by DTT. The reaction was incubated at 37 °C for 15 min, and stopped by addition of 40 µl of an ice-cold quench solution [2 % (v/v) acetic acid in ethanol, containing 1.0 mg of arachidonic acid/ml]. Then 50 µl of this mixture was spotted on Whatman LK6D TLC plates, which were developed in the organic phase of ethyl acetate/iso-octane/water/acetic acid (55:75:100:8, by vol.). Lipids were detected with I₂. Arachidonic acid was well resolved from the phospholipids, which remained at the origin. Relevant areas were scraped and counted for radioactivity by liquid scintillation. Areas corresponding to a diacylglycerol standard were also routinely scraped and counted, but contained only background radioactivity. Results are expressed as pmol of arachidonic acid released/min per mg of extraction protein. As indicated by Goldberg et al. [22], under these conditions less than 10 % of substrate was hydrolysed. Activity in the absence of cell-free extract was always less than 5 % of total activity, and was subtracted for each experiment.

To determine the effect of dephosphorylation of cPLA₂ on its enzymic activity, the 100 000 *g* supernatant fraction (60–90 µg) was incubated with 5 µg of PAP for 1 h at 30 °C [10,27]. The

resulting extent of dephosphorylation was inferred from Western blots (described below) in which the apparent molecular-mass 'shift' of cPLA₂ associated with its phosphorylation can be revealed.

Immunoblotting

Cell extracts were prepared as described above; cytosol or membrane protein (50–100 µg) was resolved on SDS/7.5 % polyacrylamide gels and blotted on to Immobilon membranes (Millipore). Blots were probed with monoclonal anti-cPLA₂ (see under 'Materials'), incubated with anti-mouse antibody linked to horseradish peroxidase, and developed by ECL.

To observe the mobility shift associated with phosphorylation of cPLA₂ [10], electrophoresis was continued at 225 V for approx. 3 h after the Bromophenol Blue dye front reached the bottom of the gel.

RESULTS

In interphase HeLa cells, histamine or thapsigargin induced the rapid Ca²⁺-dependent release of arachidonic acid

Activation of histamine H1 receptors in HeLa cells stimulates [Ca²⁺]_i elevation in a pattern that is typical of hormones that increase levels of InsP₃ [6]. As shown in Figure 1(a), in interphase cells histamine induced the rapid release of arachidonic acid.

Thapsigargin, which inhibits the Ca²⁺-ATPase that is essential for the accumulation of calcium by intracellular stores and leads to the elevation of [Ca²⁺]_i without G-protein or InsP₃ intermediates [28], also induced arachidonate release in interphase cells (Figure 1a).

Arachidonate release stimulated by histamine or thapsigargin was almost completely inhibited (93 ± 5 %; mean ± S.E.M.) by addition of 2 mM EGTA, which both blocks Ca²⁺ influx and depletes intracellular Ca²⁺ [6,8]. Preincubation with 10 µM BAPTA-AM, which leads to the intracellular accumulation of the Ca²⁺ chelator BAPTA, suppresses the normal stimulated elevation of [Ca²⁺]_i [6,29], and also inhibited (104 ± 6 %; S.E.M.) arachidonate release (results not shown). These observations are consistent with the hypothesis that release was mediated by the Ca²⁺-dependent cPLA₂. Moreover, stimulated release was decreased by nearly 80 % by the PLA₂ inhibitor mepacrine (100 µM; results not shown). This is comparable with the effects of mepacrine on the mobilization of arachidonate from keratinocytes [30] or eosinophils [31], and *in vitro*, where it has apparent effects on both the enzyme [32] and the substrate [33].

Mitotic cells did not release arachidonic acid in response to histamine or thapsigargin

In contrast with the brisk response of interphase cells, in mitotic cells stimulated release by histamine or thapsigargin was markedly suppressed (Figure 1b). The response of metaphase-arrested populations, typically only 5–10 % that of interphase, could be largely accounted for by interphase cells that contaminated the mitotic-cell preparation.

Failure of mitotic cells to release arachidonate could not be explained by the absence of increases in [Ca²⁺]_i

Since the arachidonate mobilization by interphase cells was shown to be Ca²⁺-dependent, and since the elevation of free Ca²⁺ in mitotic cells in response to histamine and thapsigargin is known to be depressed [6], the absence of a Ca²⁺ increase would seem to account, at least in part, for the inhibition of arachidonate release from mitotic cells. To test this hypothesis more directly,

Figure 1 $[^3\text{H}]$ Arachidonic acid release from interphase compared with mitotic HeLa cells stimulated with histamine or thapsigargin: role of Ca^{2+}

(a) Interphase cells: time course of $[^3\text{H}]$ arachidonic acid release from interphase cells. Labelled cells were incubated in the absence (●) or presence of histamine (20 μM ; ▲) or thapsigargin (100 nM; ■). At the times indicated, radioactivity released by 10^6 cells into the medium was determined as described in the Experimental section. Results are shown as averages of three representative experiments assayed in duplicate. (b) Mitotic cells: time course of $[^3\text{H}]$ arachidonic acid release. Mitotic cells were prepared as described in the Experimental section and incubated in the absence (○) or presence of histamine (20 μM ; △) or thapsigargin (100 nM; □). At the times indicated, radioactivity was determined and data were calculated as in (a).

we incubated cells with the Ca^{2+} ionophore A23187, in concentrations sufficient to elevate $[\text{Ca}^{2+}]$, markedly in both interphase and mitotic HeLa cells (see the Discussion section). As shown in Figure 2, addition of A23187 to interphase cells in the presence of standard medium containing high (0.4 mM) Ca^{2+} was sufficient to induce a large increase in arachidonate release. This effect of the ionophore was blocked by the addition of excess EGTA or by preincubation of cells with BAPTA-AM (results not shown). However, a parallel addition of A23187 to a suspension of mitotic cells again induced only 5–10% of the amount of arachidonate released from interphase cells (Figure 2). Thus, even when the intracellular Ca^{2+} of mitotic cells was elevated non-physiologically, mobilization of arachidonate did not occur.

Potential of arachidonate release from interphase cells, but not mitotic cells by EGF and PMA

A major regulatory process associated with the mobilization of arachidonate is called 'priming': preincubation with certain

Figure 2 Time course of A23187-stimulated $[^3\text{H}]$ arachidonic acid release from interphase and mitotic HeLa cells

Interphase (●) and mitotic (○) cells prelabelled with $[^3\text{H}]$ arachidonic acid were incubated with 1 μM A23187, and at time points indicated, radioactivity released into the medium was determined (10^6 cells/sample). Results shown are averages of three different representative experiments assayed in duplicate. Radioactivity released by both interphase and mitotic cells incubated in the absence of ionophore (results not shown) approximated to that released by mitotic cells incubated with 1 μM A23187 (○).

Table 1 Effect of PMA or EGF on $[^3\text{H}]$ arachidonate release in interphase HeLa cells

Interphase cells were labelled with $[^3\text{H}]$ arachidonic acid and resuspended in serum-free growth medium (F-12 containing 1.0 mg/ml fatty-acid-free BSA, buffered with 30 mM Na-Hepes) as described in the Experimental section. Thapsigargin (100 nM) or A23187 (1 μM) was added to cells resuspended and incubated without hormone or tumour promoter, or preincubated for 20 min with PMA (300 ng/ml) or EGF (100 nM). Thapsigargin- or ionophore-induced release of radioactivity was determined after a 30 min stimulation. Results are expressed as means $\pm$ S.E.M. of three different experiments assayed in duplicate. Virtually all of the label released was associated with non-esterified fatty acid (see the Experimental section).

Preincubation conditions	Arachidonate-mobilizing agent	Radioactivity released (c.p.m./ 10^6 cells)
Control	—	4446 $\pm$ 300
	Thapsigargin	25936 $\pm$ 1900
	A23187	17586 $\pm$ 1100
PMA	—	4752 $\pm$ 380
	Thapsigargin	42882 $\pm$ 2850
	A23187	42360 $\pm$ 2450
EGF	—	4686 $\pm$ 450
	Thapsigargin	49440 $\pm$ 3600
	A23187	38461 $\pm$ 3250

hormones that have minimal effects on arachidonate release by themselves enhances the arachidonate release induced by agents that elevate $[\text{Ca}^{2+}]$, [10,22,34]. Priming by tumour necrosis factor- α (TNF- α) has been demonstrated in HeLa cells and is correlated with phosphorylation of cPLA₂ [11]. We considered the possibility that the failure of mitotic HeLa cells to respond to Ca^{2+} might be the consequence of a block to phosphorylation that might be overcome by priming.

Figure 3 Comparison of electrophoretic mobility of interphase- and mitotic-cell cPLA₂

(a) Mobility shift of cPLA₂ in SDS/PAGE. HeLa cells were washed and resuspended in serum-free medium as described in the Experimental section, and incubated for 2 h at 37 °C. EGF (100 nM) was then added to appropriate samples, which were further incubated for 20 min. Cell extracts were prepared as described in the text, and 100 000 g-supernatant protein (75 µg/sample) was subjected to SDS/PAGE, immunoblotted with a specific monoclonal antibody to cPLA₂, and developed with ECL (see the Experimental section). Lanes 1 and 2 show that EGF induced a mobility shift; lanes 3 and 4 show that the shift was complete in mitotic cells with or without EGF. (b) Effect of phosphatase treatment on mitotic cPLA₂ mobility. For this, 100 000 g-supernatant protein (75 µg) from mitotic-cell extracts was incubated with or without 5 µg of PAP for 1 h at 30 °C, and analysed as described in (a).

When interphase cells were preincubated with EGF or PMA, and then exposed to thapsigargin or A23187, arachidonate release was increased approx. 2-fold compared with non-preincubated controls (Table 1). As shown, despite the continued presence of the priming agent, there was only a slight increase in release by EGF or PMA in the absence of Ca²⁺-elevating agents.

By contrast, in mitotic cells, neither EGF nor PMA enhanced arachidonate release in response to A23187 or thapsigargin (results not shown); release was less than 5% of the primed interphase response. Thus, agents that effectively prime interphase cells did not enable mitotic cells to respond to elevations of [Ca²⁺]_i.

By Western blotting, extracts of interphase and mitotic cells contained nearly identical amounts of cPLA₂

Immunoblot analysis of interphase and mitotic cell extracts, using a specific monoclonal anti-cPLA₂ as the primary antibody, is illustrated in Figure 3. Each lane was loaded with equal amounts of protein. From densitometry of the cPLA₂-staining region under conditions in which density was linear with dilution, it was determined that the mitotic and interphase extracts contained nearly identical amounts of cPLA₂ mass expressed per mg of protein (mitotic, 107% of interphase, average of 3 experiments). Clearly, the amount of cPLA₂ present in mitotic cells did not account for their unresponsiveness.

Mobility shift of cPLA₂

The blots also provided important information on the phosphorylation of cPLA₂ which, as discussed above, contributes

Table 2 Comparison of cPLA₂ activity from interphase and mitotic HeLa-cell extracts: effects of EGF priming

Extracts were prepared from HeLa cells preincubated with or without EGF (100 nM) for 20 min at 37 °C. cPLA₂ activity in 100 000 g-supernatant samples was determined as described in detail in the Experimental section. Values represent means ± S.E.M. for 3 determinations in 4 separate experiments. To determine the effect of dephosphorylation of cPLA₂ on its enzymic activity, extracts (60–90 µg) were incubated with 5 µg of PAP for 1 h at 30 °C before the assay. The activity in extracts incubated in parallel but without PAP was unchanged.

Condition	cPLA ₂ activity (pmol/min per mg)	
	No PAP	+ PAP
Interphase		
Control	48.79 ± 5.34	30.41 ± 2.89
+ EGF	101.69 ± 10.54	60.91 ± 6.29
Mitotic		
Control	53.50 ± 4.81	31.27 ± 2.63
+ EGF	49.92 ± 5.53	31.04 ± 3.01

to its activation [10,15,16,30]. Phosphorylated proteins characteristically are slowed in their mobility on SDS/PAGE. Several laboratories have shown that activation of cPLA₂ is associated with its decreased mobility, and that this arises from phosphorylation (e.g. [10,11,16,34]). As reported by Hoeck et al. [11] in their study of TNF-α activation of HeLa cells, we found that an appreciable fraction of the cPLA₂ in growing populations of interphase cells was present in the slower-migrating form (Figure 3a, lane 1), even when they were incubated in the absence of serum for 2 h at 37 °C. A greater proportion of the cPLA₂ was shifted to the more slowly migrating form after incubation with EGF (Figure 3, lane 2). By contrast, in mitotic cells, virtually all of the reactive material was in the slower-migrating band even before addition of hormone or ionophore (Figure 3a, lane 3). EGF did not cause a hypershift of the cPLA₂ band derived from mitotic cells (Figure 3a, lane 4). That the mobility shift was indeed the consequence of phosphorylation was supported by the effect of incubating the extract with PAP before electrophoresis. As shown in Figure 3(b), the phosphatase caused a large fraction of the mitotic PLA₂ band to shift to the more rapidly migrating species. A similar pattern was seen for interphase cPLA₂ (results not shown). Although increasing the concentration of PAP by as much as 3-fold enhanced the downward shift (results not shown), we could not find conditions in which the shift was completely reversed.

Thus the cPLA₂ of mitotic cells was present in quantity and appeared to be constitutively phosphorylated in a way that was consistent with maximal priming. However, it was possible that in mitotic cells phosphorylation occurs on a residue indifferent or even inhibitory to activation, while still leading to a mobility shift. To determine the functional state of the enzyme in mitotic cells, we examined the activity of the enzyme *in vitro*, using extracts collected in parallel with electrophoresed samples.

cPLA₂ activity in extracts of interphase cells

Extracts were made in the presence of EGTA to ensure that all enzyme was released from membrane [24], and the assay was performed on 100 000 g supernatants. Western blots showed that in fact there was little or no cPLA₂ present in the resulting cell pellets (results not shown). The assay was performed under standard conditions in high Ca²⁺ [22,24]; as noted in the Experimental section, Ca²⁺ was absolutely essential for activity. The results are shown in Table 2. The calculated activity was

comparable with that reported for CHO and other cell lines [10,22]. When interphase cells were treated with EGF immediately before preparation of the extract, activity measured *in vitro* was doubled, as reported for HER 14 cells [22]. This was approximately the same degree of stimulation measured as arachidonate release from intact interphase cells (Table 1). As discussed above, the increase in activity was associated with a decrease in cPLA₂ mobility on SDS/PAGE. In addition, phosphatase treatment decreased the activity in extracts by approx. 40% (Table 2). The loss of activity in controls after phosphatase treatment was consistent with the observed mobility shift in extracts of control cells that indicates a degree of constitutive phosphorylation.

cPLA₂ activity in extracts of mitotic cells

Surprisingly, the activity in extracts of mitotic cells was similar to that measured in extracts of interphase cells (Table 2). In fact, the activity in extracts of untreated mitotic cells, expressed per mg of cell protein, was nearly the same as that in extracts obtained from untreated (unprimed) interphase cells. Since the protein of the larger mitotic cell is, of course, greater in amount than that of the interphase cell, the activity per cell is actually greater for the mitotic.

cPLA₂ activities in samples of the phosphatase-treated mitotic samples illustrated in Figure 3(b) were decreased, like those of interphase cells, by roughly 40% (Table 2). This indicates that at least part of the cPLA₂ activity in mitotic extracts was promoted by phosphorylation. As all of the mitotic cPLA₂ was mobility-shifted, it is suggested that mitotic cPLA₂ was already 'primed'. This is consistent with the observation that preincubation of mitotic cells with EGF did not result in the stimulation of phospholipase activity measured *in vitro* (Table 2).

However, the level of 'primed' activity in mitotic extracts was no greater than in control interphase extracts. Thus it is possible that the mitotic cPLA₂ is phosphorylated, and therefore, mobility-shifted, but at site(s) that do not enhance, and may actually block, the priming of enzyme activity.

DISCUSSION

cPLA₂ is known to be activated by the synergistic actions of Ca²⁺ and specific protein phosphorylation [10,13–16]. The present paper, comparing mitotic and interphase HeLa cells, indicates the existence of yet other mechanisms of control.

We show that arachidonate mobilization in interphase HeLa cells reflects parallel phenomena described in CHO cells, fibroblasts and other cultured cell lines (e.g. [10,11,22,24,30,34]). Arachidonate release rapidly followed the addition of hormones or ionophores that raise [Ca²⁺]_i, and the release was blocked by Ca²⁺ chelators that prevent Ca²⁺ entry or buffer intracellular free Ca²⁺. These results are consistent with the known relationship of ligand-stimulated release and the actions of cPLA₂ (as opposed to the recently described non-Ca²⁺-dependent PLA₂ [35,36]). Preincubation with EGF or PMA potentiated the subsequent arachidonate release in response to agents that elevate [Ca²⁺]_i (priming). EGF priming was associated with an upward mobility shift of cPLA₂ demonstrated on SDS/PAGE. The mobility shift was at least partially reversed by phosphatase treatment. Thus interphase HeLa cells exhibited all of the regulatory phenomena currently associated with the activity of cPLA₂.

Mitotic HeLa cells, on the other hand, were remarkably refractory to all stimuli for arachidonate release, and this behaviour appears to depend on novel mechanisms. Agents that cause elevations of [Ca²⁺]_i by inositol hydrolysis (histamine), or release of Ca²⁺ from intracellular stores directly (thapsigargin),

which are powerful stimuli of arachidonate release from interphase cells, were ineffective in mitotic cells. Since arachidonate mobilization in interphase cells was strongly inhibited by Ca²⁺ chelators, and since the elevations of [Ca²⁺]_i induced by histamine and thapsigargin are inhibited in mitotic cells [6], it might be assumed that, on this basis alone, arachidonate release from mitotic cells would be inhibited. However, Figure 2 shows that, even when a calcium ionophore was used physically to convey Ca²⁺ into mitotic cells in amounts that strongly induced arachidonate release from interphase cells, release from the mitotic cells did not occur. In the process of determining the [Ca²⁺]_i [37], it was found that the addition of A23187, in the same concentration used in these experiments, to either interphase or mitotic cells caused free Ca²⁺ to rise to levels that equalled or exceeded the maximal Ca²⁺ quantifiable (> 2–3 μM).

Nonetheless, it was readily shown by Western blotting with a specific antibody to cPLA₂ that the enzyme was present in concentrations that were nearly equal in interphase and mitosis. Might the enzyme be modified in such a way that its catalytic activity is inhibited? Measurements of phospholipase activity *in vitro* suggested that this was not the case: a strong Ca²⁺-dependent activity was demonstrable in mitotic extracts that was comparable with the activity in extracts of interphase cells.

Catalysis with a membrane-vesicle substrate implies, of course, that the cPLA₂ obtained from interphase or mitotic cells was translocated to the membrane. A central question is whether the translocation that occurred *in vitro* by cPLA₂ from both interphase and mitotic extracts also occurs in intact cells. This is particularly significant, in view of the well-known reorganization of intracellular membranes that accompanies mitosis [38]. In general, when homogenates are prepared in the absence of Ca²⁺ chelators, a fraction of cPLA₂ is found associated with membranes. Indeed, Channon and Leslie [13] showed clearly that the extent of cPLA₂ association with membrane was a function of the Ca²⁺ concentration of the homogenization buffer. Consistent with this, we did find (results not shown) that, when either interphase or mitotic cells were homogenized in buffers without chelators, a fraction of cPLA₂ was associated with membrane. Thus, to measure total cPLA₂ in extracts, Ca²⁺ chelators were used to reverse enzyme translocation, so that all activity could be brought into a supernatant fraction devoid of interfering endogenous membrane. The membrane association in the absence of chelators suggests that a fraction of cPLA₂ could bind to the homogenized biological membranes of either interphase or mitotic cells, but this does not indicate that translocation actually occurs within the intact cell, in which the integrity and topological organization of membranes are preserved.

Despite the similarity in cPLA₂ activities in interphase and mitotic cells, there were important differences in cPLA₂ revealed in our studies: (1) as determined by mobility on Western blots, the amount of interphase cPLA₂ shifted was increased on preincubation with EGF; mitotic cPLA₂ was entirely shifted even before incubation with the priming agent, and (2) the activity *in vitro* of interphase cPLA₂ was enhanced by preincubation with EGF; cPLA₂ activity in mitotic extracts was unaffected by preincubation with EGF. These findings are consistent with the hypothesis that the mitotic cPLA₂ is already primed. It has been established that, in interphase cells, priming is associated with phosphorylation on Ser-505, probably mediated by MAP kinase [16]. Although MAP kinase activity appears to reach a peak in late G2 (perhaps the G2/M transition), MAP kinase activity falls to very low levels in metaphase-arrested CHO cells [39]. Assuming that metaphase-arrested HeLa cells behave similarly to CHO cells, this implies that MAP kinase reaches minimum levels at a time when cPLA₂ is heavily

phosphorylated (mobility-shifted). Several explanations for this paradox are suggested, and remain to be resolved: one is that cPLA₂ is phosphorylated by MAP kinase when cells enter mitosis, and dephosphorylation occurs only slowly as cells progress through mitosis, and another is that the mobility shift results from phosphorylation at an independent site mediated by other (perhaps mitosis-specific) protein kinases.

In any event, a cPLA₂ that is Ca²⁺-activatable in extracts, but not *in situ*, suggests a novel regulatory mechanism in mitotic cells. There may be inhibition by lipocortin [40], or some other inhibitor, that is diluted in extracts. Alternatively, there may be functional compartmentalization of cPLA₂ or its substrates related to membrane reorganization in mitotic cells [38]. These interesting possibilities remain to be investigated.

Finally, it is clear that, as for many important physiological processes, the signal pathways leading to the mobilization of arachidonate are governed by multiple layers of control that are uniquely manifest in mitotic cells. Not only is the activity of cPLA₂ in mitotic cells inhibited because elevated levels of [Ca²⁺]_i are not sustained by stimulated Ca²⁺ influx [6,7], but *in vivo*, even when Ca²⁺ is provided, arachidonic acid is not released. As arachidonate mobilization is the limiting first step in the formation of biologically active eicosanoids, mitotic cells will therefore not generate these regulatory factors.

REFERENCES

- Berlin, R. D., Oliver, J. M. and Walter, R. F. (1978) *Cell* **25**, 327–341
- Hesketh, T. R., Beaver, M. A., Rogers, J., Burke, B. and Warren, G. B. (1984) *J. Cell Biol.* **98**, 2250–2254
- Oliver, J. M., Seagrave, J. C., Pfeiffer, J. R., Feibig, M. L. and Deanin, G. G. (1985) *J. Cell Biol.* **101**, 2156–2166
- Sager, P. R., Brown, P. A. and Berlin, R. D. (1984) *Cell* **39**, 275–282
- Warren, G., Davoust, J. and Cockcroft, A. (1985) *EMBO J.* **3**, 2217–2225
- Preston, S. F., Sha'afi, R. I. and Berlin, R. D. (1991) *Cell Regul.* **2**, 915–925
- Volpi, M. and Berlin, R. D. (1988) *J. Cell Biol.* **107**, 2533–2539
- Berlin, R. D. and Preston, S. F. (1993) *Cell Calcium* **14**, 379–386
- Samuelsson, B., Dahlen, S. E., Lindgren, J. A., Rouzer, C. A. and Serhan, C. N. (1987) *Science* **237**, 1171–1176
- Lin, L. L., Lin, A. Y. and Knopf, J. L. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6147–6151
- Hoeck, W. G., Ramesha, C. S., Change, D. J., Fan, N. and Heller, R. A. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4475–4479
- Hack, N., Margolis, B. L., Ullrich, A., Schlessinger, J. and Skorecki, K. L. (1991) *Biochem. J.* **275**, 563–567
- Channon, J. Y. and Leslie, C. C. (1990) *J. Biol. Chem.* **265**, 5409–5413
- Clark, J. D., Lin, L.-L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N. and Knopf, J. L. (1991) *Cell* **65**, 1043–1051
- Qui, Z.-H., de Carvalho, M. S. and Leslie, C. C. (1993) *J. Biol. Chem.* **268**, 24506–24513
- Lin, L. L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A. and Davis, R. J. (1993) *Cell* **72**, 269–278
- Putney, J. W., Jr. (1990) *Cell Calcium* **11**, 611–624
- Brockman, J. L., Gross, S. D., Sussman, M. R. and Anderson, R. A. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 9454–9458
- Heider, H., Hug, C. and Lucocq, J. M. (1994) *Eur. J. Biochem.* **219**, 513–520
- Wheatley, D. N., Glinesk, A. and Inglis, M. S. (1988) *Cytobios* **55**, 41–50
- Espe, U., Fürstenberger, G., Marks, F., Kaszkin, M. and Kinzel, V. (1987) *J. Cancer Res. Clin. Oncol.* **113**, 137–144
- Goldberg, J. H., Viegas, M. M., Margolis, B. L., Schlessinger, J. and Skorecki, K. L. (1990) *Biochem. J.* **267**, 461–465
- Bonney, R. C., Beesley, J. S. and Franks, S. (1991) *J. Reprod. Fertil.* **93**, 449–460
- Bonventre, J. V., Gronich, J. H. and Nemenoff, R. A. (1990) *J. Biol. Chem.* **265**, 4934–4938
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Clark, J. D., Milona, N. and Knopf, J. L. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7708–7712
- Kramer, R. M., Roberts, E. F., Manetta, J. V., Hyslop, P. A. and Jakubowski, J. A. (1993) *J. Biol. Chem.* **268**, 26796–26804
- Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R. and Dawson, A. P. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2466–2470
- Tsien, R. Y. (1980) *Biochemistry* **19**, 2396–2404
- Kast, R., Fürstenberger, G. and Marks, F. (1993) *J. Biol. Chem.* **268**, 16795–16802
- White, S. R., Strek, M. E., Kulp, G. V. P., Spaethe, S. M., Burch, R. A., Neely, S. P. and Leff, A. R. (1993) *J. Clin. Invest.* **91**, 2118–2125
- Vigo, C., Lewis, G. P. and Piper, P. J. (1980) *Biochem. Pharmacol.* **29**, 623–627
- Jain, M. K., Streb, M., Rogers, J. and DeHaas, G. H. (1984) *Biochem. Pharmacol.* **33**, 2541–2551
- Durstin, M., Durstin, S., Molski, T. F. P., Becker, E. L. and Sha'afi, R. I. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3142–3146
- Radmanadham, S., Gorss, R. W., Han, X. and Turk, J. (1993) *Biochemistry* **32**, 337–346
- Ackerman, E. J., Kempner, E. S. and Dennis, E. A. (1994) *J. Biol. Chem.* **269**, 9227–9233
- Gryniewicz, G., Peonie, M. and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Lucocq, J. M., Berger, E. G. and Warren, G. (1989) *J. Biol. Chem.* **109**, 463–474
- Tamemoto, H., Kadowaki, T., Tobe, K., Ueki, K., Izumi, T., Chatani, Y., Kohno, M., Kasuga, M., Yazaki, Y. and Akanuma, Y. (1992) *J. Biol. Chem.* **267**, 20293–20297
- Glaser, K. B., Mobilio, D., Chang, J. Y. and Senko, N. (1993) *Trends Pharmacol. Sci.* **14**, 92–98