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Maximal epidermal growth-factor-induced cytosolic phospholipase A_2 activation *in vivo* requires phosphorylation followed by an increased intracellular calcium concentration

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The 85 kDa cytosolic phospholipase A_2 ($cPLA_2$) preferentially catalyses the hydrolysis of arachidonic acid from the *sn*-2 position of phospholipids. $cPLA_2$ can be activated by extracellular stimuli such as thrombin, platelet-derived growth factor and epidermal growth factor (EGF). A full activation of $cPLA_2$ requires an increase of intracellular Ca^{2+} concentration and phosphorylation on Ser-505 by mitogen-activated protein (MAP) kinase. Because EGF can provoke an increase in intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and activation of MAP kinase, we investigated the role of these pathways in EGF-induced activation of $cPLA_2$. Characterization of two cell lines expressing different numbers of EGF receptors (HERc13 and HER14) revealed that both were activating MAP kinase in response to EGF, but only HER14 responded with an increase in $[Ca^{2+}]_i$. In this study we used both cell lines as a tool to clarify the role of each pathway in $cPLA_2$ activation. We show that EGF stimulates $cPLA_2$ activity in both cell lines *in vitro* as measured in cytosolic fractions, but only in HER14 *in vivo* as measured by ^3H release from cells prelabelled with $[^3H]$arachidonic acid. This latter activation can be restored in HERc13 cells by the addition of the ionophore A23187. Interestingly, this effect is only observed when EGF stimulation precedes A23187 addition. The phosphorylation of MAP kinase, however, was identical under identical conditions. We conclude that a maximal $cPLA_2$ activation by EGF requires both, and in this order: MAP kinase activation followed by a rise in $[Ca^{2+}]_i$ concentration.

INTRODUCTION

Phospholipase A_2 (PLA_2) family members catalyse the hydrolysis of the ester bond at the *sn*-2 position of membrane phospholipids [1]. This reaction can result in arachidonic acid formation, which is the rate-limiting step in the biosynthesis of arachidonic acid metabolites such as prostaglandins and leukotrienes. These eicosanoids act in important biological processes such as mitogenic signalling [2] and cell movement [3]. The Ca^{2+}-dependent PLA_2 family can be divided into the low-molecular-mass secreted PLA_2 ($sPLA_2$) and the high-molecular-mass cytosolic PLA_2 ($cPLA_2$). The latter is found in a variety of cells and tissues, including rat mesangial cells [4,5], the human monoblast U937 cell line [6–9] and fibroblasts [10,11]. Furthermore, this 85 kDa $cPLA_2$ is optimally active at micromolar concentrations of calcium and is arachidonyl-selective [8], so it is likely to be involved in the formation of prostaglandins and leukotrienes.

A full activation of $cPLA_2$ is dependent on the presence of calcium. The calcium directs the $cPLA_2$ to membranes and for this translocation process the Ca^{2+}-dependent phospholipid-binding domain of the enzyme is essential [8,9,12]. In several cell lines the $cPLA_2$ becomes phosphorylated on extracellular stimuli such as thrombin, epidermal growth factor (EGF) and platelet-derived growth factor [12–16]. Recently, it has been shown *in vitro* that the serine/threonine-specific mitogen-activated protein (MAP) kinase is able to phosphorylate PLA_2 on serine residue 505 [15,16], and interestingly this phosphorylation is identical to the site of agonist-induced phosphorylation *in vivo* [15–17]. By site-directed mutagenesis it was shown that MAP kinase-mediated phosphorylation of Ser-505 is required for agonist-induced arachidonate release [15].

EGF is a potent mitogen that initiates a signal transduction cascade by binding to a specific 170 kDa tyrosine kinase receptor [18–21]. The tyrosine kinase activity is essential for all receptor-mediated responses involved in mitogenic signalling [19,22]. The wide variety of EGF-induced responses includes a rise in $[Ca^{2+}]_i$, MAP kinase activation, PLA_2 activation and the release of arachidonic acid metabolites [12,14,23–26]. *In vitro*, maximal activation of $cPLA_2$ requires both certain concentrations of Ca^{2+} and phosphorylation of Ser-505 by MAP kinase. Because EGF activates MAP kinase and raises intracellular $[Ca^{2+}]$ by two independent signal transduction pathways, it is tempting to speculate that both these responses contribute to an activation of $cPLA_2$ by EGF.

When characterizing two cell lines, denoted HERc13 and HER14, we were unable to detect the EGF-induced release of arachidonic acid in HERc13 cells, in contrast to HER14 cells. We show below that both cell lines are able to bind EGF with similar K_d values. However, EGF stimulation in HER14 leads to MAP kinase phosphorylation and increased intracellular calcium concentration, whereas the HERc13 displays only MAP kinase phosphorylation and no increase in intracellular Ca^{2+}. These findings provide a model system in which it is possible to discriminate between the effect of $[Ca^{2+}]_i$ and MAP kinase in EGF-dependent activation of $cPLA_2$. Using these two cell lines we demonstrate that phosphorylation of $cPLA_2$ precedes Ca^{2+}-

Abbreviations used: $cPLA_2$, cytosolic phospholipase A_2; DG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; FCS, foetal calf serum; $[Ca^{2+}]_i$, intracellular $[Ca^{2+}]$; MAP, mitogen-activated protein.

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dependent translocation in the EGF-induced activation process of $cPLA_2$.

EXPERIMENTAL

Cell culture

In this study we used NIH 3T3 fibroblasts devoid of endogenous EGF-receptors [3T3(0)] and HERc13 and HER14 fibroblasts, which are mouse 3T3(0) cells transfected with the human EGF-receptor cDNA expressing approximately 65000 and 250000 EGF receptors per cell, respectively. The cells used are routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% (v/v) foetal calf serum (FCS) (Gibco, Breda, The Netherlands) for 3T3(0) and 7.5% (v/v) FCS and 250 μg/ml geneticin (G418) for transfected cells, buffered with 40 mM $NaHCO_3$ under a 7.5% CO_2 atmosphere. For the experiments, subconfluent cultures of fibroblasts were grown in six-well or twelve-well tissue culture clusters or 75 or 165 cm^2 flasks (Costar) and kept for the last 16 h in serum-free medium.

$cPLA_2$ activity assay

Cells were stimulated with 200 ng/ml EGF (receptor grade, Biomedical Technologies, Stoughton, MA, U.S.A.). After the appropriate incubation periods the medium was discarded and cells were washed twice with cold Ca^{2+}- and Mg^{2+}-free PBS. Subsequently, the cells were scraped in homogenization buffer containing 50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1 mM PMSF, 5 mM benzamidine, 50 mM NaF, 250 μM Na_3VO_4, 10 mM Na_2HPO_4, 1 μM leupeptin and 1 μM aprotinin, and disrupted with ten strokes of a Potter–Elvehjem motor-driven Teflon pestle at 600 rev./min. The supernatant fraction was prepared by centrifugation at 15000 ***g*** for 20 min at 4 °C. The $cPLA_2$ activity in these extracts was measured by the release of radiolabelled arachidonic acid from the *sn*-2 position of 1-stearoyl-2-[1-^{14}C]arachidonoyl phosphatidylcholine (Amersham International, Amersham, Buckinghamshire, U.K.). The radiolabelled phosphatidylcholine was dried under nitrogen and then dispersed in water by sonication for 3×20 s under nitrogen. The assay incubation mixture contained, in a total volume of 200 μl, 0.2 M Tris/HCl, pH 8.5, 1 mM free Ca^{2+} in excess of EDTA, 5 μM phosphatidylcholine substrate and approximately 25 μg of protein. After incubation for 5 min at 37 °C the reaction was stopped and the released [1-^{14}C]arachidonic acid was extracted by a modified Dole extraction procedure [27]. Under these assay conditions, less than 5% of the substrate was hydrolysed and the reaction was linear with time for 5 min.

Arachidonic acid release

To measure arachidonic acid release from intact fibroblasts, the cells were prelabelled by a 48 h incubation with 0.5 μCi [^{3}H]arachidonic acid (Amersham) in 12- or 24-well plates. After labelling, the cells were washed twice with DMEM with 0.01% (w/v) BSA (fatty acid-free), preincubated with DMEM containing 0.01% (w/v) BSA (fatty acid-free) in the absence of serum for 2.5 h and then stimulated as indicated in the Figure legends. After the incubations the media were collected and centrifuged for 5 min at 14000 ***g***. The radioactivity in the supernatant was determined in a liquid-scintillation counter.

Binding experiments

EGF was iodinated by the chloramine-T method, yielding specific activities of 500000–900000 c.p.m. per ng EGF (EGF receptor grade from Collaborative Research, Waltham, MA, U.S.A.; ^{125}I from New England Nuclear, Boston, MA, U.S.A.) as described previously [28,29]. Cells were grown to 80% confluency and incubated for 3 h at 4 °C with 1 ml mixtures of labelled and unlabelled EGF in binding medium [DMEM, 0.1% (w/v) BSA, 25 mM Hepes, pH 7.4]. The cells were washed twice with ice-cold PBS, dissolved in 1 ml of 1M NaOH and radioactivity was determined by counting in a gamma-counter (Crystal 5410 Multi detector RIA system, United Technologies Packard). Non-specific binding was determined by using a 500-fold excess of unlabelled EGF. For Scatchard analysis, the binding data were analysed with the LIGAND program [30] as described previously [29].

MAP kinase phosphorylation

Cells were either not stimulated or stimulated with different concentrations of EGF or for different periods with EGF as indicated in the legends of the Figures. Then the medium was discarded and cells were washed twice with ice-cold PBS. The cells were dissolved in hot sample buffer [31] and proteins were separated on a 12.6% (w/v) SDS/polyacrylamide gel with an acrylamide/bisacrylamide ratio of 167:1 on the Miniprotean II system of Bio-Rad (Veenendaal, The Netherlands) at 100 V. After electrophoresis, proteins were blotted onto nitrocellulose (Schleicher and Schuell, Dassal, Germany) and MAP kinase was detected by a polyclonal anti-ERK2 antibody (kindly provided by Dr. J. L. Bos, University of Utrecht) or monoclonal anti-ERK2 (UBI, Lake Placid, NY, U.S.A.) and the enhanced chemiluminescence (ECL) detection system (Amersham).

Measurements of $[Ca^{2+}]_i$

Nearly confluent monolayers of cells, starved for 16 h in medium with 0.5% (v/v) FCS, were loaded with indo-1 by incubating them with 10 μM indo-1 acetoxymethyl ester at 37 °C for 45 min in Hepes-buffered saline (140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2.0 mM $CaCl_2$, 10 mM D-glucose, 10 mM Hepes, pH 7.40). The cells were washed three times with Hepes-buffered saline and mounted in a chamber. Indo-1 fluorescence was measured with a Perkin Elmer LS 3000 fluorescence spectrometer. Experiments were conducted at 33 °C. $[Ca^{2+}]_i$ was determined, with an excitation wavelength of 355 nm and an emission wavelength of 405 nm, by standard procedures [23,25]

RESULTS

EGF activates PLA_2 *in vivo* in HER14 but not in HERc13 cells

During the course of our investigation on the mechanism of $cPLA_2$ activation by EGF *in vivo* [14] we observed that one particular NIH 3T3 fibroblast cell line expressing transfected EGF receptors, designated HERc13, failed to release arachidonic acid in response to EGF, in contrast to another cell line, designated HER14. In the present experiment HERc13 and HER14 cells were prelabelled with [^{3}H]arachidonic acid, then stimulated with EGF; ^{3}H release into the medium was measured. As shown in Figure 1, EGF was ineffective in HERc13 cells in increasing the amount of ^{3}H release, whereas under the same conditions EGF did induce an increase in ^{3}H release from HER14 cells. Under the same conditions, no increase in intracellular ^{3}H-labelled fatty acid level was observed in HERc13 cells as determined by TLC (results not shown). As described previously, the release of [^{3}H]arachidonic acid can be used as a measure for $cPLA_2$ activity [13]. Because both cell lines originated from the same NIH 3T3 cells, and both are transfected with the

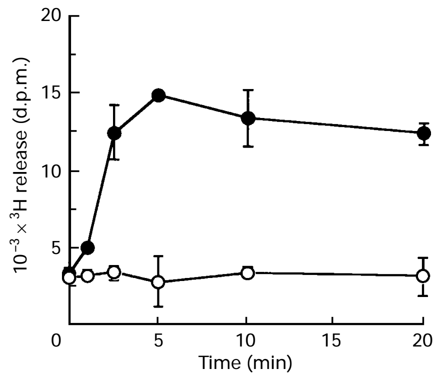

Figure 1 Time course of EGF-stimulated ^3H release from HERc13 and HER14 cells

HERc13 (○) and HER14 (●) cells were labelled with [^3H]arachidonic acid (0.5 μCi per well) for 48 h, and then washed twice with DMEM/BSA, preincubated with DMEM/BSA without serum for 2.5 h and stimulated with 200 ng/ml EGF for the indicated periods. Media were collected and centrifuged at 14000 ***g*** for 5 min. The radioactivity in the supernatant was determined and the results are expressed as the means ± S.E.M. for five separate experiments, each performed in duplicate.

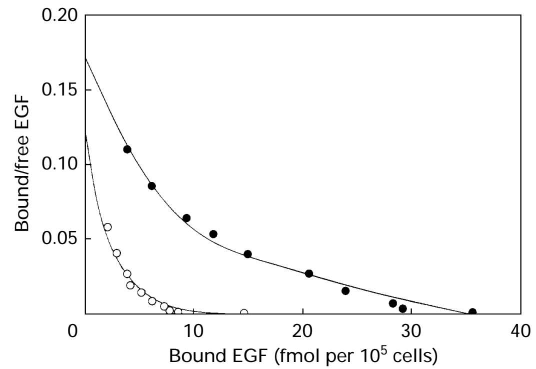

Figure 2 Scatchard analysis of HERc13 (○) and HER14 (●) cells

Cells were incubated with 0.5 ng/ml ^{125}I-labelled EGF and increasing concentrations of unlabelled EGF (0.5–500 ng/ml) in binding medium (DMEM/Hepes/BSA) at 4 °C for 3 h. Cells were washed twice with PBS and were subsequently lysed in 1 M NaOH. Radioactivity was determined and analysed by the method of Scatchard.

Table 1 $cPLA_2$ activity *in vitro* in cytosolic fractions

Cells were either non-treated (control) or treated with 200 ng/ml EGF for 5 min at 37 °C and then lysed. Cytosolic fractions were prepared by centrifugation at 15000 ***g***. $cPLA_2$ activity in the supernatant was determined by the release of [^{14}C]arachidonic acid from the *sn-2* position of 1-stearoyl-2-[1-^{14}C]arachidonoyl phosphatidylcholine. Stimulation results are shown as means ± S.E.M.

Cell line	PLA_2 activity ($nmol \cdot min^{-1} \cdot mg^{-1}$) Control	+EGF	EGF stimulation (% of control)
3T3(0)	2.22	2.21	100 ± 5 ($n = 6$)
HERc13	2.07	4.06	196 ± 10 ($n = 3$)
HER14	1.30	2.61	201 ± 6 ($n = 6$)

same cDNA for human EGF receptor, the difference observed seems to be of particular interest. One reason for the absence of the EGF-induced ^3H release from HERc13 cells could be a defect or absence of $cPLA_2$ in this particular cell line. Therefore we measured the $cPLA_2$ protein levels in cell lysates from 3T3(0), HERc13 and HER14 cells by immunoblotting with a polyclonal antibody raised against $cPLA_2$. In these cell lines $cPLA_2$ protein was detected to be the same (results not shown). Subsequently, the EGF-induced PLA_2 activation was studied *in vitro*. Cell lysates of HERc13 and HER14 cells, either non-stimulated or stimulated with EGF for 5 min, were assayed as described in the Experimental section. As shown in Table 1, in HERc13 and HER14 cells EGF treatment increased the $cPLA_2$ activity to the same extent. No increase was observed in non-transfected NIH 3T3 cells [3T3(0)], which were used as a negative control. These data clearly demonstrate that HERc13 cells do have $cPLA_2$, which can be activated by EGF treatment. The reason for the absence of EGF-induced $cPLA_2$ activity *in vivo* in HERc13 cells therefore seems to be altered properties in the signal transduction cascade leading to $cPLA_2$ activation.

HERc13 and HER14 cells both bind EGF and induce MAP kinase phosphorylation, but differ in EGF-induced $[Ca^{2+}]_i$ increase

To establish the cause of the inability of HERc13 cells to activate $cPLA_2$ *in vivo* we characterized both cell lines with respect to EGF-induced signal transduction. First the EGF binding characteristics of both cells were determined: cells were incubated with different concentrations of ^{125}I-EGF. After washing, specific binding was determined and the data were analysed by the Scatchard method, as described in the Experimental section. For both cell lines this reveals a curvilinear plot (Figure 2) indicating the presence of EGF-receptors of high and low affinities, with K_d values of 0.07–0.09 nM and 1.1–2.4 nM respectively, and receptor numbers of 250000 and 65000 per cell for HER14 and HERc13 respectively. These data indicate that both cell lines bind EGF with the same affinity.

To determine EGF-induced signal transduction in HERc13 and HER14 cells, we stimulated both cell lines with different EGF concentrations and for different periods with a fixed EGF concentration, and determined the phosphorylation of MAP kinase. MAP kinase is a key enzyme in several signal transduction cascades and phosphorylates a diverse set of substrates, such as p62TCF, RS6-kinase and $cPLA_2$ [15,32,33]. On EGF stimulation, MAP kinase becomes phosphorylated on threonine and tyrosine residues [34,35]. This results in altered mobility in SDS/PAGE. As shown in Figure 3a, in both cell lines EGF induces a shift of MAP kinase on the gel, which is visible at low EGF concentrations and reaches a maximum at 50 ng/ml EGF. The time course reveals a maximal MAP kinase phosphorylation at 5 min of EGF exposure in both cell lines followed by a gradual decline in phosphorylation from 15 to 60 min in both cell lines (Figure 3b). Furthermore, in HERc13 cells only 50% of the MAP kinase is shifted in contrast to a total shift in HER14 cells. This difference between the two cell lines may be caused by the difference of the number of EGF receptors in the two cell lines. No EGF-induced MAP kinase phosphorylation was observed in 3T3(0) cells (results not shown). Because EGF-induced MAP kinase phoshorylation occurs in both cell lines at the same EGF concentration and with the same time dependence, this pathway cannot explain the inability of EGF to activate $cPLA_2$ in these cells.

Another signal transduction pathway that may be involved in the induction of $cPLA_2$ activation is initiated by phospholipase

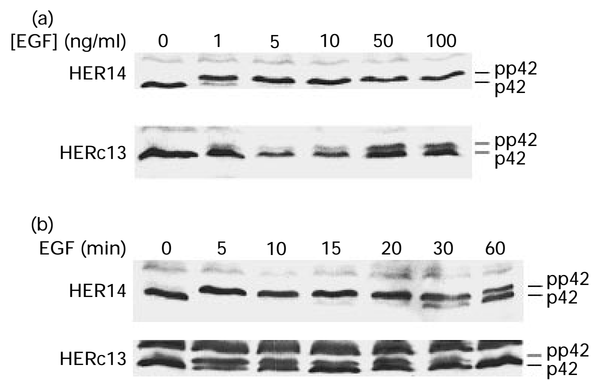

Figure 3 EGF-induced p42MAP kinase phosphorylation of HERc13 and HER14 cells

(**a**) Serum-starved cells were incubated with 0, 1, 5, 10, 50 and 100 ng/ml EGF for 5 min at 37 °C, washed twice with ice-cold PBS and lysed in hot sample buffer. Proteins were separated on a 12.6% (w/v) denaturing SDS gel (acrylamide:bisacrylamide 167:1) and blotted on nitrocellulose. MAP kinase was detected with an anti-ERK2 antibody and revealed with ECL. Non-phosphorylated (p42) and phosphorylated (pp42) MAP kinase bands are indicated. (**b**) Serum-starved cells were incubated with 5 ng/ml EGF for 0, 5, 10, 15, 30 and 60 min at 37 °C, washed twice with ice-cold PBS and lysed in hot sample buffer. Analysis of MAP kinase phosphorylation was performed as in (**a**).

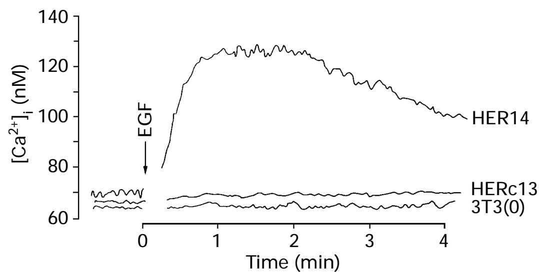

Figure 4 EGF-induced changes in $[Ca^{2+}]_i$ in 3T3(0), HERc13 and HER14 cells

Serum-starved cells were loaded with indo-1 by incubating with 10 μM indo-1 acetoxymethyl ester at 37 °C for 45 min in Hepes-buffered saline. Cells were washed with Hepes-buffered saline and mounted in a chamber. Indo-1 fluorescence was measured with a Perkin Elmer LS 3000 fluorescence spectrometer at 33 °C at an excitation wavelength of 355 nm and an emission wavelength of 405 nm.

C-γ1 (PLC-γ1). This enzyme, which is also activated by the EGF receptor [36,37], hydrolyses phosphatidylinositol 4,5-bisphosphate into the two second messengers diacylglycerol (DG) and *myo*-inositol 1,4,5-trisphosphate (IP_3). The second messenger IP_3 causes a rise in intracellular Ca^{2+} concentration. $cPLA_2$ activation is known to be dependent on the mobilization of intracellular Ca^{2+}. Because EGF is capable of increasing the intracellular Ca^{2+} concentration [23], we investigated this process in HERc13 and HER14 cells. For this purpose cells were loaded with the fluorescent indicator Indo-1 to monitor changes in $[Ca^{2+}]_i$ after EGF treatment. As shown in Figure 4, in HER14 cells EGF induces an increase in $[Ca^{2+}]_i$, reaching a maximum in 2 min, followed by a gradual decline to control levels. In contrast, in HERc13 cells the Ca^{2+} signal is absent. As expected, no EGF-induced increase in $[Ca^{2+}]_i$ was observed in 3T3(0) cells. The inability of EGF to induce an increase in $[Ca^{2+}]_i$ in HERc13 cells might be the cause of the inability of EGF to induce $cPLA_2$ in these cells.

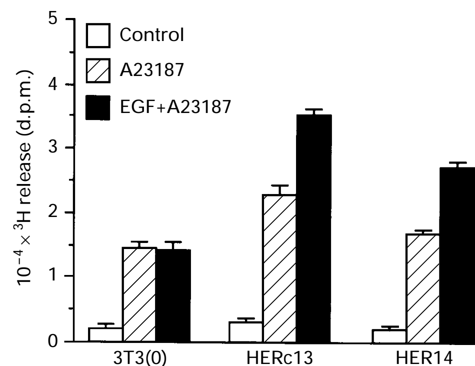

Figure 5 Effect of EGF and A23187 on ^{3}H release from 3T3(0), HERc13 and HER14 cells

The cells were labelled with [^{3}H]arachidonic acid as described in the legend to Figure 1 and then treated with medium alone (control), incubated with 1 μM A23187 (A23187) for 5 min or stimulated with EGF (200 ng/ml) for 5 min followed by A23187 incubation for 5 min (EGF + A23187). The ^{3}H released into the media was measured by scintillation counting. Data are expressed as the means $\pm$ S.E.M. for six experiments.

EGF-induced arachidonic acid release from HERc13 cells *in vivo* can be observed by ionophore A23187 addition after the EGF treatment but not before

The above results demonstrate clearly that HERc13 cells lack the EGF-induced increase in intracellular Ca^{2+}, and it is tempting to suggest that the absence of the Ca^{2+} signal is related to the absence of EGF-induced $cPLA_2$ activation in HERc13 cells (Figure 1). Moreover, because the $cPLA_2$ is found to be activated by EGF in HERc13 cell lysates by the assay *in vitro* in which Ca^{2+} is added, Ca^{2+} seems to play an important role in the $cPLA_2$ activation *in vivo*. To confirm this hypothesis we tried to obtain a $cPLA_2$ response in HERc13 cells *in vivo*: we used the ionophore A23187 to provoke an increase in $[Ca^{2+}]_i$ independent of EGF. HERc13, HER14 and 3T3(0) cells were labelled with [^{3}H]arachidonic acid, then were left non-stimulated or stimulated with EGF, followed by the addition of A23187. As a control, cells were left non-stimulated, receiving neither EGF nor A23187. As shown in Figure 5, A23187 induced an increase in ^{3}H release to the same extent in the three cell types on its own. However, pretreatment of these cells with EGF resulted in a further increase in ^{3}H release in HERc13 cells compared with A23187 alone. This same effect was also measured in HER14 cells, whereas in 3T3(0) cells no effect of EGF was observed. Therefore the inability of HERc13 to respond to EGF by a release of arachidonic acid is possibly due to the inability of these cells to provoke a raise in intracellular Ca^{2+} on EGF stimulation.

Next we used the ability of A23187 to generate arachidonic acid release in HERc13 cells as a tool to study the temporal relationship between $cPLA_2$ phosphorylation [12] and a rise in $[Ca^{2+}]_i$ on $cPLA_2$ activation. To do this, we first determined the influence of A23187 on EGF-induced MAP kinase phosphorylation in the two cell lines: both cell lines were stimulated with A23187 or EGF alone, or with EGF for increasing periods combined with A23187 treatment before or afterwards. As shown in Figure 6, A23187 alone does not induce (HERc13), or partly induces (HER14), MAP kinase phosphorylation, whereas EGF alone induces a maximal MAP kinase phosphorylation. Furthermore EGF-induced MAP kinase phosphorylation still occurs after A23187 pretreatment. In addition, A23187 post-treatment does not affect the EGF-induced MAP kinase phosphorylation state.

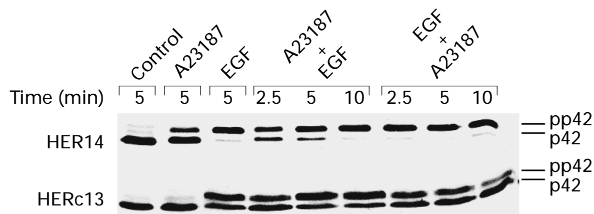

Figure 6 Time-dependent MAP kinase phosphorylation by EGF in the presence of A23187

Serum-starved HERc13 and HER14 cells were either non-stimulated or stimulated with EGF (200 ng/ml) or A23187 (1 μM) alone, or with EGF (200 ng/ml) for 2.5, 5 or 10 min in combination with a pretreatment or post-treatment with A23187 (1 μM) at 37 °C. At the appropiate time, cells were dissolved in hot sample buffer. MAP kinase phosphorylation was analysed as described in the legend to Figure 3, except that here a monoclonal ERK2 antibody was used.

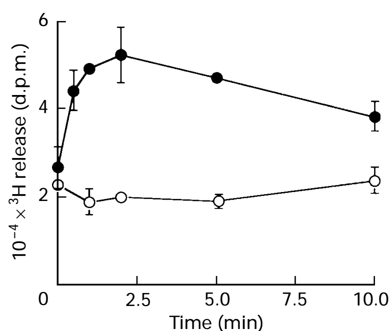

Figure 7 Time-dependent increase in ^{3}H release by EGF in the presence of A23187

HERc13 cells were labelled with [^{3}H]arachidonic acid as described in the legend to Figure 1 and stimulated with 1 μM A23187 for 5 min followed by 200 ng/ml EGF for the time indicated (○), or with EGF (200 ng/ml) for the indicated time followed by 1 μM A23187 for 5 min (●). Data are expressed as the means ± S.E.M. for three experiments.

Subsequently the $cPLA_2$ activation was determined with the same EGF–A23187 combinations. Cells were again prelabelled with [^{3}H]arachidonic acid, then were either stimulated with EGF for different periods followed by A23187 addition for 5 min, or incubated with A23187 for 5 min first and then stimulated with EGF for different periods. As shown in Figure 7, EGF induces ^{3}H release when the cells were stimulated with EGF followed by an incubation with A23187. However, when HERc13 cells were pretreated with A23187 before EGF stimulation, no effect of EGF was observed. Similar results were obtained in HER14 cells (results not shown). These results indicate that the phosphorylation of $cPLA_2$, probably by MAP kinase, has to precede a rise in $[Ca^{2+}]_i$. Intracellular Ca^{2+} then acts in the translocation of the $cPLA_2$ to membranes [12].

DISCUSSION

Stimulation of arachidonic acid release has been found in many cells expressing EGF receptor [38–41], and arachidonic acid and its metabolites have been shown to be involved in mitogenic responses. The production of arachidonic acid can be accomplished by sequential activation of PLC and DG lipase, but the liberation of arachidonic acid is regulated most directly by PLA_2.

In a previous study [14] we demonstrated that EGF-induced activation of arachidonate release is still detectable after partial purification of the enzyme, indicating that PLA_2 is the enzyme involved in the liberation of arachidonic acid. With biochemical characteristics resembling those of previously purified and cloned $cPLA_2$s [8,9,42], the EGF-stimulated PLA_2 was identified as a $cPLA_2$ [14]. Recently we have shown that EGF induces serine phosphorylation of this $cPLA_2$, which is required for its EGF-induced activation. Furthermore, it was shown that the EGF-induced rise in $[Ca^{2+}]_i$ results in a translocation of $cPLA_2$ from cytosol to membranes. In this process the second messenger Ca^{2+} is coupled to $cPLA_2$ activation [8,12,24]. The $cPLA_2$ translocation is most probably directed by its Ca^{2+}-dependent phospholipid binding domain [8].

In the present study we have further elucidated the role of two EGF-induced pathways most probably involved in the activation of $cPLA_2$ *in vivo*: one in which MAP kinase is involved, which is shown to be capable of $cPLA_2$ phosphorylation *in vitro* [15], and the second in which EGF induces a Ca^{2+} influx resulting in a $cPLA_2$ translocation from cytosol to membranes [12]. EGF-induced activation of the $cPLA_2$ in HERc13 cells, as measured in a cell-free extract, is approximately 2-fold, comparable with HER14 cells. This effect is most probably due to activation of the $cPLA_2$ by phosphorylation because we showed previously that this activation can be reversed by treatment with alkaline phosphatase [12]. EGF-induced MAP kinase phosphorylation displays the same dose- and time-dependent characteristics for both cell lines. In contrast, EGF was unable to increase the $[Ca^{2+}]_i$ in HERc13 cells. As a result, no EGF-induced ^{3}H release from intact cells was measured. Raising $[Ca^{2+}]_i$ by using the Ca^{2+} ionophore A23187, which has no effect on EGF-induced MAP kinase phosphorylation, is required for the complete activation of $cPLA_2$ by EGF in HERc13 cells. The same phenomenon was observed in an epithelial cell line where transforming growth factor-α-induced PLA_2 activation requires the influx of Ca^{2+} [43]. The inability of EGF to cause an increase in $[Ca^{2+}]_i$ could be related to the number of EGF receptors in this particular cell type, and this implies that the relationship between a $[Ca^{2+}]_i$ increase and the number of EGF receptors is not linear. A 4-fold decrease in the Ca^{2+} signal of HER14 cells, which contain four times more EGF receptors than HERc13 cells, would still be detectable with the system used. Furthermore, a rise in $[Ca^{2+}]_i$ can be detected in cells containing roughly the same amount of EGF receptors as HERc13 cells [44]. Taken together, the EGF-induced increase in the release of arachidonic acid and its metabolites is due to both an increase in the enzyme activity of the $cPLA_2$, as measured in the assay *in vitro*, and a calcium-dependent translocation to membranes, as measured in the experiments *in vivo* [12]. Furthermore, we show that the phosphorylation of $cPLA_2$ has to precede a calcium-induced translocation to membranes to obtain a total activation of the enzyme by EGF. Apparently, phosphorylation of $cPLA_2$, probably by MAP kinase, cannot take place at the position where the $cPLA_2$ action occurs, which is at membranes. After EGF stimulation, MAP kinase becomes active in the cytoplasm and then even translocates to the nucleus [45].

Our results are also of particular interest in view of the role for arachidonic acid and its metabolites in mitogenic signalling. It has been demonstrated that arachidonic acid metabolism is involved in EGF-induced c-*myc* and c-*fos* expression in fibroblasts and mesangial cells, respectively [2,46,47]. Despite of the lack of EGF-induced arachidonic acid release, EGF does induce DNA synthesis and cell division in HERc13 cells (results not shown), implying that these two responses are not entirely dependent on arachidonic acid metabolism.

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