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Lipocortin 1 and the Control of $cPLA_2$ Activity in A549 Cells

GLUCOCORTICOIDS BLOCK EGF STIMULATION OF $cPLA_2$ PHOSPHORYLATION

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ABSTRACT. Epidermal growth factor (EGF) rapidly stimulates the release of arachidonic acid in A549 cells by a mechanism that is sensitive to pertussis toxin [1]. We show that EGF treatment of A549 cells stimulates phosphorylation of cytosolic phospholipase A_2 ($cPLA_2$) through a mechanism that is similarly inhibited by pertussis toxin. The level of $cPLA_2$ expression is, apparently, not changed during this period. Pretreatment of cells with dexamethasone (10–100 nM) for 3 hr prevents this activation of $cPLA_2$ by EGF, without changing the level of $cPLA_2$ expression. The effect of dexamethasone is reversed in the presence of the neutralizing antilipocortin Mab 1A but not by the nonneutralizing antilipocortin 1 control Mab 1B. This strongly suggests that lipocortin 1 mediates the effect of dexamethasone by inhibiting activation of $cPLA_2$. This concept is supported by the fact that a peptide Lc13-25 (10–200 μg/mL), derived from the N-terminus of lipocortin 1, also inhibits activation of $cPLA_2$ by EGF in these cells. BIOCHEM PHARMACOL 52;2:351–356, 1996.

KEY WORDS. annexin 1; MAPK; growth factor signal transduction; arachidonic acid; G protein

EGF† stimulates proliferation of the A549 human lung adenocarcinoma cell line by increasing the release of autocrine modulators, such as PGE_2 [2, 3] following activation of arachidonic acid release [1]. A549 cells do not express $sPLA_2$ activity [4, 5], and the involvement of either PLC or PLD also appears to be an unlikely mechanism of generating arachidonic acid [1, 4]. The discovery of a cytosolic form of PLA_2 in U937 cells, that exhibits a more selective preference for arachidonyl-containing phospholipids and a low Ca^{2+} requirement [6–8], was confirmed by us to also be expressed in A549 cells [4]. This form of the enzyme is now widely accepted to be the most important mediator of receptor-coupled arachidonic acid release [9].

$cPLA_2$ expression is enhanced by long-term (>10 hr) treatment with proinflammatory cytokines [10] and, because release of arachidonic acid is the rate limiting step in eicosanoid generation, this is likely to be responsible for the increased prostaglandin release seen after cytokine treatment. This induction of $cPLA_2$ expression is repressed by dexamethasone and is clearly one of several important mechanisms that account for the inhibitory actions of glucocorticoids. However, $cPLA_2$ activity is also acutely regulated by rapid phosphorylation/dephosphorylation reactions over short time periods (<1 hr) [10]. The nonphosphorylated and less active form is converted to a phosphorylated and more active form following treatment of cells with growth factors or cytokines [10, 11], and this, clearly, permits a more responsive control over arachidonic acid release. These two forms of the enzyme are revealed as closely spaced doublet bands (approximately 85 kDa) under the appropriate electrophoretic conditions, and conversion from one form to the other is seen as changes in the intensity of the bands relative to one another. EGF induced phosphorylation of $cPLA_2$ is now known in some circumstances to be mediated by MAPK [12], which is, itself, activated by a cascade of kinases ultimately activated by $p21^{ras}$ [13, 14]. This mechanism helps to explain the rapid agonist activation of $cPLA_2$ activity without the need to increase expression of the enzyme.

EGF stimulation of arachidonic acid release in this cell line is inhibited by dexamethasone in a process mediated by the induction of lipocortin 1 [1]. We show here that this is accomplished by inhibiting the phosphorylation and, thus, the activation of $cPLA_2$ rather than by regulating its expression.

MATERIALS AND METHODS

Materials

EGF and Pertussis toxin were from Sigma (Poole, U.K.). RU486 was a gift from Roussel-Uclaf (Romainville, France). The well-characterized [15] monoclonal antibodies 1A and 1B to lipocortin 1 were provided by Dr. J.

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† *Abbreviations:* EGF, epidermal growth factor; $cPLA_2$ cytosolic phospholipase A_2; $sPLA_2$ secretory phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; MAPK, mitogen-activated protein kinase; GR, glucocorticoid receptor; GRE, glucocorticoid response element; Mab, monoclonal antibody; PGE_2, prostaglandin E_2; Ser, serine; Tyr, tyrosine.

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Browning (Biogen, Boston). The specific rabbit polyclonal antibody to $cPLA_2$ was raised against peptide fragment 726–796 haptenized with KLH by Dr. Clive Jackson (Astra Research, Loughborough, U.K.). Lipocortin 1-derived peptides were synthesised by Dr. G. Schnorrenberg, Boehringher Ingelheim KG, D-6507 Ingelheim am Rhein, Germany, and purified by HPLC. The sequences were validated by FAB-MS. The peptides were prepared as N-acetyl derivatives to enhance biological stability as: Peptide Lc1-12 (MAMVSEFLKQAW) and Peptide Lc13-25 (FIENEEQEYVQTV). Peptides and growth factors were stored and dispensed in siliconized plasticware.

Cell Culture

A549 cells (Flow) were maintained in continuous log phase growth in Dulbecco's Modified Eagles Medium/F-12 (DMEM/F-12) containing Phenol Red and 10% foetal calf serum (FCS) in T-150 flasks (Greiner Glos, U.K.). The cells were not allowed to reach confluence at any time because this diminishes their response to growth factors. Cultures were routinely checked for the absence of mycoplasma contamination. To provide sufficient material for blotting, experiments were performed in T-150 flasks seeded at 3×10^6 cells/flask in the above media. After 24 hr, the media was changed to DMEM/F12 without Phenol Red or serum and left for a further 24 hr. The media was changed again to DMEM/F12 containing growth factor, steroid, or antibody for the appropriate incubation period. The culture media was aspirated and the cells washed with PBS, 1 mM EDTA. The monolayer was lysed in 1.5 mL PBS, 10 mM EDTA, 1% Triton T-X100, 1 mM PMSF, 1 mM sodium orthovanadate, and 0.01% leupeptin. Samples were stored in multiple aliquots at −70°C.

Western Blotting of $cPLA_2$

The protein concentration of each sample was determined by Bradford assay, and total protein equivalents loaded in each lane. SDS-PAGE was performed according to the method of Laemmli, but using 10 × 8 cm gels with 12% acrylamide, overun by 2 hr after the dye front reached the bottom. Color markers (Sigma) were used as a guide to cut out the appropriate portion of the gel for transfer to Immobilon PVDF membrane (Millipore, Watford, U.K.). Membranes were blocked with 5% dried milk fat powder in PBS, 0.1% Tween-20 for 1 hr at 4°C. The primary antibody to $cPLA_2$ was used at 1:4000 and incubated with blots overnight. After washing the membrane, goat antirabbit HRP conjugate (Sigma, U.K.) was used at 1:2000 for 2 hr, then, washed again and the signal was detected with DAB.

Image Analysis

Western blots were directly scanned using a ScanMaker (Microtek, Redondo Beach, CA) and the image composite transferred into Power Point (Microsoft, Seattle, WA) running on an Apple Macintosh for printing. Densitometric analysis was performed with NIH Image 1.54. Individual blots were enlarged to enable the upper and lower bands of $cPLA_2$ to be easily separated and their respective intensities determined. The values given are only semiquantitative and are meant to give some relative numerical guide to the *ratio* of band intensities.

RESULTS

EGF Stimulates $cPLA_2$ Activity via G Protein Activation

In the experiment in untreated A549 cells reported in Fig. 1, $cPLA_2$ migrates as a doublet, with the higher molecular weight species forming 71% of the total and the lower molecular weight species forming 29%. In contrast, cells treated for 1 hr with 10 nM EGF, $cPLA_2$ migrates predominantly as the higher molecular weight species (85%) whereas the lower molecular weight species forms only 15% of the total (Fig. 1). The stimulation by EGF over control varied between 14–35%, (Mean 23.2 ± SD 7.8%, $P < 0.01$, $n = 5$, Student t-test (unpaired) subjected to Bonferonni correction). Pre-treatment with pertussis toxin (1–100 ng/mL) for 3 hr inhibits this conversion in a dose-dependent manner (Fig. 1). The ratio of band intensities is plotted in

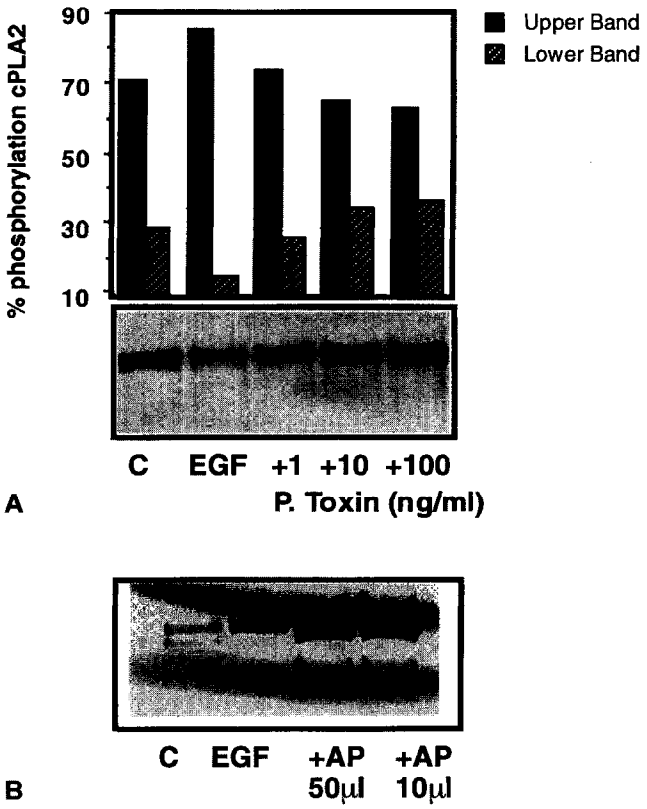

FIG. 1. Activation of $cPLA_2$ is inhibited by pertussin toxin. (A) A549 cells were pretreated with pertussin toxin for 3 hr prior to stimulation with 10 nM EGF for 1 hr. The relative intensities of the doublet bands are plotted in the histogram. (B) EGF stimulation is reversed following incubation of the lysed cell preparation with alkaline phosphatase (µL/mL acrylic beads, Sigma, 30 min, 37°C, pH 9).

the histogram above the blot to illustrate in numerical form the change in activation state. In all the following figures only the % value of the lower molecular weight species is given, from which the value of the upper band can be calculated. The % ratio in untreated cells did vary from experiment to experiment, presumably due to variations in basal stimulation of the cells; this is reflected in variations in basal arachidonic acid release [1]. EGF and dexamethasone always provoke the same effect, even though the base line may not be the same in each experiment. To confirm that the observed band shifts were due to phosphorylation, EGF-treated samples were incubated with alkaline phosphatase immobilized on acrylic beads at 37°C for 30 min at pH 9, after which the lower molecular weight species reappears (Fig. 1). The upper phosphorylated band does not completely disappear, however, as also found by others [9], presumably indicating the presence of an irreversibly phosphorylated pool of the enzyme. This would also explain the basal phosphorylation seen in control cells (all Figs.).

Activation of cPLA$_2$ is Blocked by Dexamethasone

Pretreatment of A549 cells with 100 nM dexamethasone prevents EGF (10 nM, 1 hr)-induced phosphorylation of cPLA$_2$ in a time-dependent manner (Fig. 2). The inhibition is optimal after 3 hr, when the band ratios have returned to control levels. This time-course parallels the induction of lipocortin 1 [3] and suppression of arachidonic acid release [1] in these cells. The effect is GR mediated because co-incubation with a 10-fold excess is the antagonist RU486 prevents the action of dexamethasone (Fig. 2). We have, previously, shown RU486 prevents dexamethasone induction of lipocortin 1 and suppression of arachidonic acid release [1, 3]. This new data is consistent with the notion that glucocorticoids directly inhibit cPLA$_2$ activation. These results are typical of at least 3 such experiments.

Dexamethasone Inhibition of cPLA$_2$ is Mediated by Lipocortin 1

Pretreatment with dexamethasone in the range 1 nM–1 μM (data not shown) for 3 hr inhibits EGF (10 nM, 1 hr)

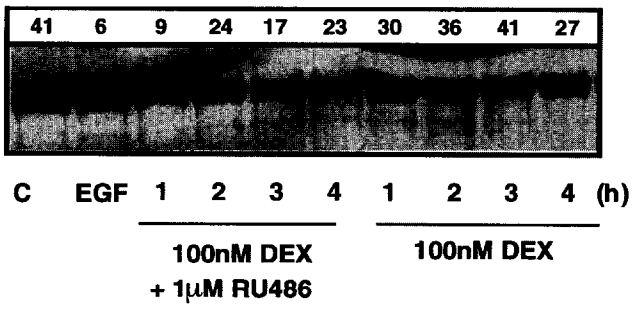

FIG. 2. Activation of cPLA$_2$ is inhibited by dexamethasone. A549 cells were pretreated with 100 nM dexamethasone or 100 nM dexamethasone + 1 μM RU 486 for 3 hr prior to stimulation with 10 nM EGF for 1 hr. Numbers in panel represent % expression of lower (unphosphorylated) band.

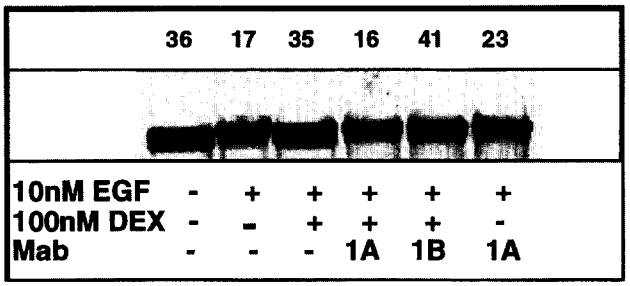

FIG. 3. Dexamethasone inhibition of cPLA$_2$ is mediated by lipocortin 1. A549 cells were pretreated for 3 hr with 100 nM dexamethsone ± Mabs at 5 μg/mL prior to stimulation with 10 nM EGF for 1 hr. 1A, neutralizing ab; 1B, nonneutralizing ab. Numbers in panel represent % expression of lower (unphosphorylated) band.

stimulation of cPLA$_2$ in a dose-dependent manner. The inhibitory effect of the steroid at 100 nM (and lower doses not shown) is completely reversed by co-incubation with a neutralizing antilipocortin 1 Mab 1A (5 μg/mL) (Fig. 3). The neutralizing properties of this antibody are well characterized [15]. We have, previously, demonstrated that Mab 1A reverses dexamethasone suppression of arachidonic acid release in A549 cells, whereas the nonneutralizing antilipocortin 1 Mab 1B does not [1]. In agreement with this data, we now show that Mab 1B (5 μg/mL) does not reverse dexamethasone (100 nM, 3 hr) inhibition of cPLA$_2$ (Fig. 3). These results are typical of at least 3 such experiments, and support our original hypothesis that glucocorticoids inhibit arachidonic acid release by a lipocortin 1-dependent inhibition of cPLA$_2$ activity [1].

An N-terminal Fragment of Lipocortin 1 Inhibits cPLA$_2$ Activation

Pretreatment of A549 cells with peptide Lc13-25 (3 hr) inhibits activation of cPLA$_2$ by EGF (10 nM, 1 hr) in the dose range 10–200 μg/mL (5.4–108.6 μM) (Fig. 4). We have, previously, shown that this peptide inhibits EGF stimulation of arachidonic acid release and cell growth [1, 2]. The effect appears to depend on an unhindered tyrosine residue at position 21 because its substitution results in a loss of activity [1, 2]. Furthermore, the other half of the N-terminus, Lc1-12, is much less (<100-fold) active in this model [1, 2]. We confirm that peptide Lc1-12 (10–200 μg/mL, 6–122.2 μM) is much less effective in inhibiting cPLA$_2$ activation (Fig. 4) in these cells. These results are typical of at least 3 such experiments.

DISCUSSION

Glucocorticoids inhibit arachidonic acid release [1], but the precise mechanism by which this is accomplished has been debated [16]. This glucocorticoid action is blocked by actinomycin D, strongly suggesting that the effect is mediated *via* the synthesis of an inhibitory protein(s). Several groups independently identified and isolated such a putative in-

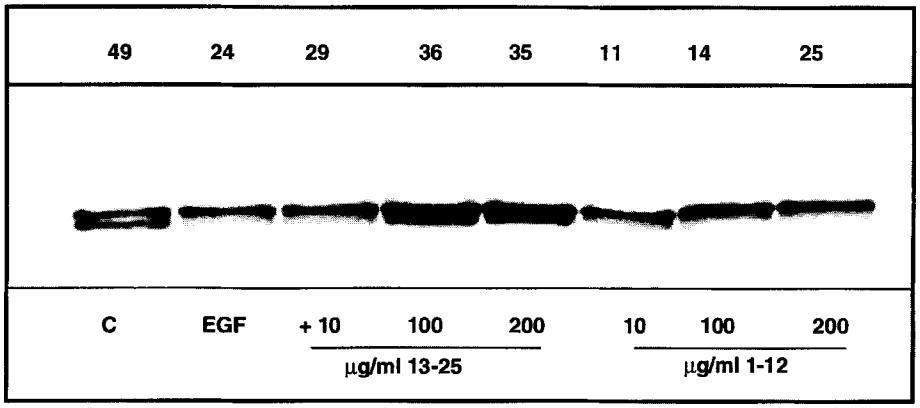

FIG. 4. Peptide Lc13-25 inhibits activation of $cPLA_2$. A549 cells were pretreated for 3 hr with peptide Lc1-12 or Lc13-25, prior to stimulation with 10 nM EGF for 1 hr. Numbers in panel represent % expression of lower (unphosphorylated) band.

hibitor—now termed lipocortin 1. This protein has potent anti-inflammatory and antiproliferative properties. [3, 17] Lipocortin 1 was originally proposed to function by inhibiting PLA_2 [18]. However, subsequent work with the 14 kDa porcine pancreas PLA_2 showed that the inhibitory effect seemed to be caused by "sequestration" of phospholipid substrate, rather than by direct inhibition of the enzyme [16].

The discovery of a novel, high-molecular-weight cytosolic form of PLA_2 [4, 6–8] that is activated in a G protein-dependent manner (Fig. 1) has helped to clarify the cellular mechanisms involved in regulating arachidonic acid release. Stimulation of arachidonic acid release by EGF is inhibited by pertussis toxin [1, 19], and we show here that pertussis toxin inhibits the phosphorylation and, thus, activation of $cPLA_2$ (Fig. 1). This confirms, first, that it is this form of PLA_2 that is coupled to the EGF-receptor signal transduction cascade [20] and, second, provides direct evidence for a G protein-dependent activation.

Activation of $cPLA_2$ is inhibited in a time-dependent manner (max effect at 3 hr) by dexamethasone (Fig. 2) that parallels inhibition of arachidonic acid release [1]. Moreover, during this time, the gross level of $cPLA_2$ expression remains unchanged because apparently only the degree of activation is inhibited. Induction of $cPLA_2$ expression in fibroblasts is blocked by dexamethasone [10], perhaps because the promoter for $cPLA_2$ contains a glucocorticoid response element (GRE, [21]). However, we provide evidence, here, for a further level of control whereby glucocorticoids can block activation of pre-existing $cPLA_2$ protein, and that the *rapid* release of arachidonic acid following EGF treatment is related more to the state of activation of $cPLA_2$ than to the level of expression. Dexamethasone blocks this rapid release of arachidonic acid by inhibiting the activation of $cPLA_2$ and this provides another important mechanism of action of glucocorticoids, in addition to regulating $cPLA_2$ gene expression. However, dexamethasone does not inhibit basal activation of $cPLA_2$ (Figs. 2 and 3), again, perhaps indicating the existence of a resistant pool of the enzyme.

Several strands of evidence indicate that dexamethasone acts through the induction of lipocortin 1 to the cell surface to inhibit arachidonic acid and PGE_2 release and cell growth [1–3, 15]. We show that the antilipocortin 1 Mab 1A inhibits the dexamethasone inhibition of $cPLA_2$ activation, whereas a nonneutralizing antilipocortin 1 Mab 1B does not (Fig. 3). All of these observations clearly imply not only that lipocortin 1 mediates the glucocorticoid effect but, also, that this level of control is manifested by directly regulating the activity of the cytosolic form of PLA_2.

This concept is further supported by the fact that the peptide Lc13-25 derived from the N-terminus of lipocortin 1 also inhibits activation of $cPLA_2$ (Fig. 4). This peptide mimicks the effect of glucocorticoids and native lipocortin 1 in inhibiting cell growth, arachidonic acid release, and PGE_2 release [1, 2]. It, therefore, seems likely that these effects can be explained by an inhibition of $cPLA_2$ activation by this peptide. The peptide Lc1-12 does not inhibit arachidonic acid or PGE_2 release or cell growth [1, 2] and also does not affect $cPLA_2$ activation (Fig. 4). Peptide Lc13-25 is derived from the N-terminal portion of lipocortin 1 that does not bind phospholipid. Therefore, it seems unlikely that Lc13-25 could sequester phospholipid substrate in the manner proposed for inhibition of the 14 kDa porcine pancreas form of PLA_2 [18]. This mechanism of inhibition is, in any case, apparently not a feature of the cytosolic form of PLA_2 [22]. The nature of these assays inevitably makes it very difficult to interpret accurately whether a mechanism of inhibition is a direct effect or an influence on substrate. The band shift assay we have used here provides a more direct mechanism for evaluating $cPLA_2$ activity, and could provide a useful method for screening inhibitors of activation of this form of the enzyme.

Binding of EGF to its receptor activates intrinsic Tyr kinase activity [23], yet $cPLA_2$ is phosphorylated on a serine residue [9]. This implies that intermediary kinase(s) are involved in the activating mechanism. Phosphorylation of

$cPLA_2$ on Ser 505 is now known, in some circumstances, to be mediated by MAPK [12]. This enzyme is, itself, activated by both Tyr and Ser/Thr kinases [24] and may serve to co-ordinate these signalling pathways. MAPK is a key mediator of cell proliferation, presumably through its ability to activate nuclear transcription factors [25]. Under other circumstances, however, $cPLA_2$ can be activated by pathways not mediated by MAPK [26]; there are, presumably, as yet undiscovered kinases involved and we cannot rule out the possibility of inhibition of one of these pathways. In our system, the activity of peptide Lc13-25 is critically dependent on the Tyr^{21} residue because substitution of this with nonphosphorylatable analogues results in loss of activity [1, 2]. It is, therefore, possible that this peptide acts by blocking a Tyr kinase event in the signalling pathway leading to activation of MAPK or other mediating kinases.

MAPK is activated by a sequence of kinases linked to $p21^{ras}$ [27]. Activation of $p21^{ras}$ by EGF receptor is mediated by a number of adaptor proteins, such as Sos and Grb2 [28]. The binding interactions between these proteins are through so-called SH2 domains, in which a Tyr residue plays a central function [29]. Recently, the catalytic specificity of the amino acid sequences around this Tyr residue has been determined [30]. The optimal peptide substrate for EGF receptor is **E-E**-E-**E-Y**-F-E-L-**V** [30]; the corresponding sequence of peptide Lc13-25 is **E-E**-Q-**E-Y**-V-Q-T-**V**. Therefore, another mechanism of action of this peptide could involve disruption of SH2 binding. The precise mechanism/s of how exogenously applied peptides can influence intracellular signalling events is beyond the scope of this paper. However, we have shown that ^{125}I-labelled peptides from lipocortin 1 are internalized in an energy-dependent manner by human neutrophils [31].

We provide evidence, here, for a mechanism that explains how glucocorticoids inhibit the rapid release of arachidonic acid by inhibiting *activation* of $cPLA_2$. This phenomenon is mediated by lipocortin 1, which appears to act by inhibiting a component of the signalling mechanism. These results do not detract from the conventional notions of glucocorticoids reducing expression of $cPLA_2$. Nor do we claim that lipocortin 1 is the sole mediator because glucocorticoids are known to influence over 1% of the genome but, rather, these results demonstrate an additional level of control to account for the rapid events of steroid hormones.

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