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LIPOCORTIN-1 AND THE CONTROL OF ARACHIDONIC ACID RELEASE IN CELL SIGNALLING

GLUCORTICOIDS INHIBIT G PROTEIN-DEPENDENT ACTIVATION OF $cPLA_2$ ACTIVITY

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Abstract—In pre-labelled A549 cells epidermal growth factor (EGF) (10 nM) stimulates the release of [5,6,8,9,11,12,14,15-^{3}H(N)]-arachidonic acid (^{3}H-AA) by approximately 70%. Increasing Ca_i^{2+} with thapsigargin (50 nM) stimulates ^{3}H-AA release by approximately 120%. However, the combined use of these two agents results in a synergistic stimulation of ^{3}H-AA release by over 700%. The EGF stimulated release is sensitive to pertussis toxin (10 ng/mL) and guanosine 5′-*O*-(2-thiodiphosphate) suggesting a G protein-mediated event. This is supported by the fact that the G protein activators AlF_4^- and guanosine 5′-*O*-(2-thiotriphosphate) both stimulate ^{3}H-AA release. The stimulation of ^{3}H-AA release by both EGF or direct G protein activation is completely blocked following pre-treatment for 3 hr with 1 nM dexamethasone. This effect is reversed with a neutralizing antibody to lipocortin-1 (1 μg/mL) suggesting that this protein mediates the inhibitory effects of glucocorticoids on agonist activated ^{3}H-AA release. Thapsigargin stimulation of ^{3}H-AA release is insensitive to dexamethasone treatment. A peptide fragment from the N-terminus of lipocortin-1-Lc13–25 (20–200 μg/mL) mimics the effect of glucocorticoid in suppressing both EGF and G protein activated ^{3}H-AA release. A peptide with Me-Tyr substituting Tyr^{21} is much reduced in activity suggesting that the presence of this residue is essential. As peptide Lc13–25 is not derived from the Ca^{2+}/phospholipid binding domain of the native protein then sequestration of phospholipid substrate for PLA_2 remains an unlikely mechanism of action for this peptide.

Key words: annexin-1; EGF; thapsigargin; A549 cells; MAP kinase; tyrosine kinase

In the A549 human lung adenocarcinoma cell line PGE_2 functions as an autocrine modulator of cell growth [1]. Growth factors such as EGF§ stimulate PGE_2 release and thus the proliferation of A549 cells [2]; similar effects have been described in BALB/c3T3 cells [3]. Glucocorticoids such as dexamethasone which inhibit the release of PGE_2 also inhibit A549 cell proliferation, a process that is mediated by induction of the protein lipocortin-1 [1]. The cyclooxygenase inhibitor indomethacin also inhibits A549 cell proliferation [1] and blocks EGF mitogenesis [3] both effects of which can be restored by the addition of exogenous PGE_2 [1, 3]. Lipocortin-1 was originally proposed to function by inhibiting the activity of PLA_2 [4] and thus the supply of arachidonic acid for metabolism by the cyclooxygenase pathway to PGE_2. Recently, a novel cytosolic, highly selective, high molecular weight form of (c)PLA_2 has been described in U937 cells [5–7] and also by us in A549 cells [8]. This form of PLA_2 is now thought to mediate growth factor activation of arachidonic acid release [9]. A549 cells do not express $sPLA_2$ activity [8, 10] and immunoblotting of cell extracts failed to detect any $sPLA_2$ expression whereas $cPLA_2$ was found to be present [10]. Furthermore, the release of arachidonic acid from the concerted action of PLC and DAG lipase does not appear to play a major role in these cells [8]. Thus, it seems that the mechanisms employed by A549 cells to generate arachidonic acid are probably solely dependent on $cPLA_2$. In this study we investigate the possibility that lipocortin-1 is a key regulatory factor in the signal transduction systems which release arachidonic acid by $cPLA_2$ activation. We describe here the G protein-mediated regulation of arachidonic acid release in A549 cells by EGF and the involvement of lipocortin-1 as an inhibitor of the signal transduction sequence leading to $cPLA_2$ activation in these cells.

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§ Abbreviations: PLA_2, phospholipase A_2; $cPLA_2$, cytoplasmic PLA_2; $sPLA_2$, secretory PLA_2; PLC, phospholipase C; DAG, diacyl-glycerol; PKC, protein kinase C; IP_3, inositol triphosphate; MAPK, mitogen-activated protein kinase; GTPγS, guanosine 5′-*O*-(2-thiotriphosphate); GDPγS, guanosine 5′-*O*-(2-thiodiphosphate); AlF_4^-, 25 nM NaF + 10 μM $AlCl_3$; Ca_i^{2+}, intracellular calcium; EGF/thapsigargin, 10 nM epidermal growth factor/50 nM thapsigargin; ^{3}H-AA, [5,6,8,9,11,12,14,15-^{3}H(N)]-arachidonic acid.

MATERIALS AND METHODS

Materials. Genestein and methyl 2,5-dihydroxy-

cinnamate were from Gibco. The DAG-lipase inhibitor RG80267 was from Rhône-Poulenc-Rorer. ^{3}H-AA (3700 GBq/mmol) was from NEN. Monoclonal antibodies to lipocortin-1 were generously provided by Dr J. Browning (Biogen). All other agents were from Sigma, U.K. Peptides and growth factors were stored and dispensed in siliconized plastic ware.

Cell culture. A549 cells (Flow) were maintained in continuous log phase growth in DMEM/F-12 containing phenol red and 10% FCS in T-150 flasks (Greiner). The cells were not allowed to reach confluence at any time as this diminishes their response to growth factors. The cells were routinely checked for the absence of mycoplasma contamination.

Measurement of arachidonic acid release. Subconfluent cells were seeded into 12-place multi-well plates (Falcon) at 3×10^5 cells/mL/well in DMEM/F-12, 10% FCS and incubated overnight. ^{3}H-AA in ethanol was evaporated to dryness under N_2 and resuspended in an appropriate volume of DMEM/F-12 (w/o phenol red) and after vortex mixing left at 37° for 1 hr. After washing the cells with PBS, 9.25 kBq of ^{3}H-AA in 0.5 mL DMEM/F-12 (w/o phenol red) was added to each well and incubated overnight. The media containing free ^{3}H-AA was then removed and the cells washed three times with 1 mL DMEM/F-12 containing 1 mg/mL BSA. The cells thus labelled with ^{3}H-AA were then treated with test agents or vehicle controls diluted into DMEM/F-12 (w/o phenol red) for the indicated times. After incubation, 0.4 mL of medium was removed from each well for scintillation counting. In experiments using guanine nucleotide analogues or neomycin it was necessary to permeabilize the cells prior to addition of the test agents by treating with saponin (40 μg/mL) in DMEM/F-12 for 3 min [11, 12].

Peptide preparation. All peptides were kindly synthesized by Dr G. Schnorrenberg (Boehringer 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 thus: Peptide Lc1–12 (MAMVSEFLKQAW), Peptide Lc13–25 (FIENEEQEYVQTV), Peptide Lc21–33 (YVQTVKSSKGGPG) and Peptide Lc13–25(Tyr-Me^{21}) (FIENEEQE[Y-Me]VQTV).

Statistical analysis. All experiments were performed in triplicate (N = 3) and each experiment presented is a typical example of at least three such experiments. Results are expressed as the mean ± 1 SD and presented as percentage changes. All statistical calculations were performed on the raw numerical data of the experiments presented. Student's *t*-test (unpaired) with the Bonferonni Correction to allow for multiple testing within each group was used to determine statistical significance with $P < 0.001$ as the nominal value for significance.

RESULTS

EGF and thapsigargin synergistically stimulate arachidonic acid release

Treatment of A549 cells for 30 min with 10 nM EGF alone increases ^{3}H-AA release by up to 70% above control levels (Fig. 1A). However, treatment for a further 30 min with thapsigargin synergistically stimulates ^{3}H-AA release by up to 700% above control levels in a dose-dependent manner, with an EC_{50} of 50 nM (Fig. 1A). Similarly, treatment with 50 nM thapsigargin alone for 30 min stimulates ^{3}H-AA release by over 100% above control levels (Fig. 1B). However, pre-treatment with EGF for 30 min synergistically stimulates ^{3}H-AA release in a dose-dependent manner up to 400% above control levels (Fig. 1B). The effect of thapsigargin on ^{3}H-AA release is time dependent (Fig. 1C). Treatment of cells with 10 nM EGF followed by 50 nM thapsigargin increases release up to 30 min but declines back to control levels by 120 min presumably as Ca_i^{2+} stores are depleted. The effect of EGF and thapsigargin on ^{3}H-AA release is sensitive in a dose-dependent manner to pre-treatment with dexamethasone (Fig. 1D). Stimulation by 10 nM EGF and 50 nM thapsigargin (abbreviated to EGF/thapsigargin hereafter) is significantly reversed by 10^{-10} M dexamethasone (65.8%, $P < 0.001$) and concentrations above following a 1 hr pre-treatment, and by 10^{-12} M dexamethasone (69.9%, $P < 0.001$) and concentrations above following a 3 hr pre-treatment. This sensitivity by A549 cells to dexamethasone is similar to that which we have previously reported for inhibition of cell growth [1].

Dexamethasone inhibits EGF stimulated release of arachidonic acid via the induction of lipocortin-1

Pre-treatment of A549 cells with 5 nM dexamethasone for 3 hr completely inhibits EGF (10 nM) stimulated release of ^{3}H-AA (*$P < 0.001$), whereas thapsigargin (50 nM) stimulated release is not significantly affected (Fig. 2A). Therefore the reversal of EGF/thapsigargin stimulated release by dexamethasone appears to be predominantly at the level of the EGF pathway. This inhibition by dexamethasone of ^{3}H-AA release is reversed by the anti-glucocorticoid RU486 (50 nM, *$P < 0.001$) indicating that this is a receptor mediated effect (Fig. 2B). Pre-treatment of A549 cells with 1 nM dexamethasone for 3 hr significantly (*$P < 0.001$) inhibits the EGF/thapsigargin stimulation of ^{3}H-AA release and this effect is reversed by co-incubation with 1 μg/mL of the neutralizing anti-lipocortin-1 mAb 1A (Fig. 2C). Co-incubation with 1 μg/mL of the non-neutralizing anti-lipocortin-1 mAb 1B has no significant effect on the dexamethasone suppression of ^{3}H-AA release (Fig. 2D). The effects are dose dependent as the suppression of ^{3}H-AA release by 2 nM dexamethasone is significantly (*$P < 0.001$) reversed by 2 μg/mL of mAb 1A but not by 1 μg/mL (Fig. 2C), again mAb 1B has no effect (Fig. 2D).

EGF/thapsigargin stimulates arachidonic acid release via a glucocorticoid sensitive G protein-dependent pathway

Pertussis toxin inhibits EGF/thapsigargin stimulated release of ^{3}H-AA in a dose-dependent manner between 0.001 and 10 ng/mL (data not shown). Pre-treatment of A549 cells with 10 ng/mL pertussis toxin for 3 hr significantly inhibits EGF (10 nM)

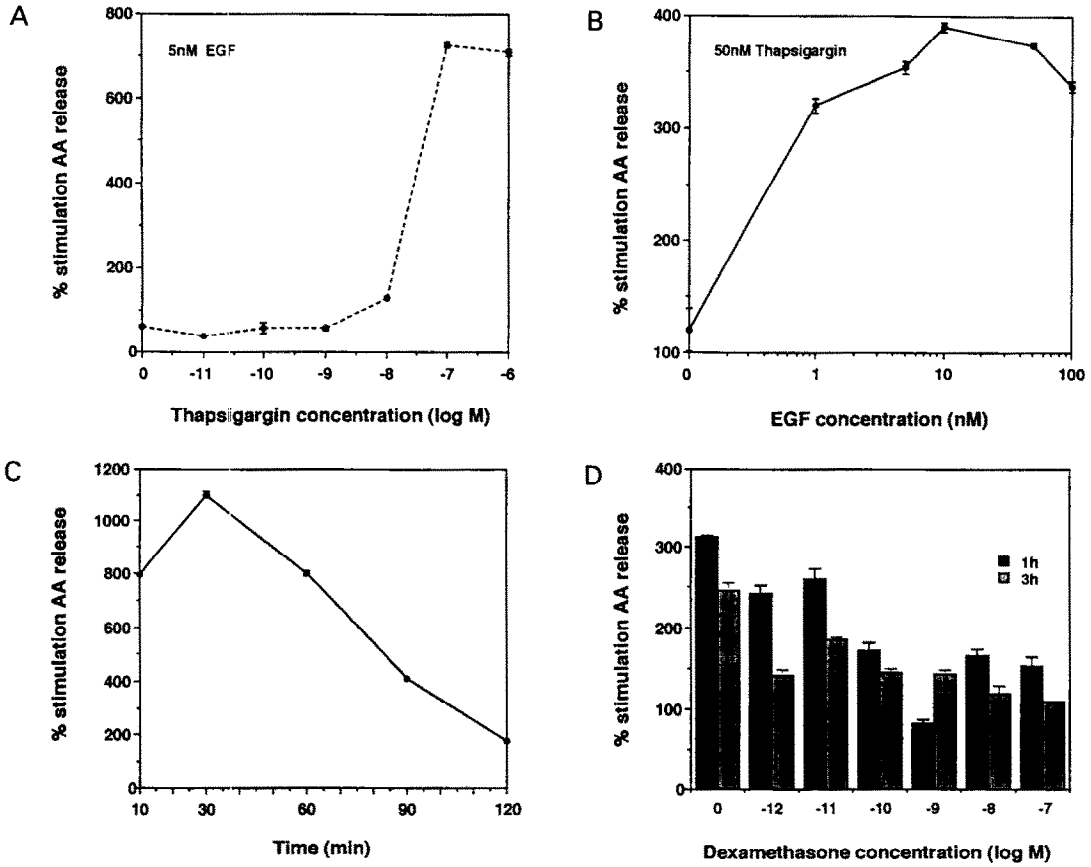

Fig. 1. EGF and thapsigargin synergistically stimulate the release of ^{3}H-AA in A549 cells. A, 10 nM EGF stimulates ^{3}H-AA release after 30 min but treatment with thapsigargin for a further 30 min synergistically stimulates release (10 nM and above). B, 50 nM thapsigargin stimulates ^{3}H-AA release after 30 min but pre-treatment for 30 min with EGF synergistically stimulates release (1 nM and above). C, Treatment with 10 nM EGF for 30 min followed by a time course with 50 nM thapsigargin. D, Dexamethasone inhibits the stimulation of ^{3}H-AA release by 10 nM EGF/50 nM thapsigargin when added 1 and 3 hr before treatment. Each point is the mean of 3 wells ± 1 SD.

stimulated release of ^{3}H-AA (*$P < 0.001$), whereas thapsigargin (50 nM) stimulated release is not significantly affected (Fig. 3A). Therefore the reversal of EGF/thapsigargin stimulated release by pertussis toxin also appears to be predominantly at the level of the EGF pathway. Pre-treatment of A549 cells with 40 μg/mL saponin for 3 min is essential to allow entry of guanine nucleotide analogues. The permeabilization process itself releases ^{3}H-AA, therefore stimulated release is calculated as a percentage change from the appropriate saponin control. We found that pre-treatment of permeabilized A549 cells with 100 μM GDPβS for 30 min prior to treatment with EGF, thapsigargin or their combination significantly inhibited EGF (*$P < 0.001$) but not thapsigargin stimulation of ^{3}H-AA release (Fig. 3B).

Conversely, 100 μM GTPγS stimulated ^{3}H-AA release above control levels in a time-dependent manner, with a maximum stimulation at 30 min (data not shown) as has been previously reported [11, 13]. Following dexamethasone treatment for 3 hr, and after permeabilization for 3 min, A549 cells were treated for 30 min with GTPγS and ^{3}H-AA release measured. GTPγS (100 μM) stimulated ^{3}H-AA release by 30% and this was blocked by pre-treatment with dexamethasone in a dose-dependent manner (1–10 pM, data not shown). The effect of 5 pM dexamethasone was significantly reversed by 50 pM RU486 (*$P < 0.001$) suggesting a receptor mediated event (Fig. 3C). The cell permeable AlF_4^- anion is also a G protein activator that stimulates ^{3}H-AA release [13]. This is generated by incubating A549 cells in DMEM culture media with 25 nM NaF and 10 μM $AlCl_3$ for 1 hr. AlF_4^- stimulated ^{3}H-AA release by over four-fold above control levels and this was significantly (*$P < 0.001$) inhibited by pre-treatment with 5 nM dexamethasone for 3 hr (Fig. 3D). The inhibition by dexamethasone was significantly (*$P < 0.001$) reversed by co-incubation with 50 nM RU486 (Fig. 3D) again suggesting that the effect is receptor mediated.

Arachidonic acid release is not affected by inhibitors of PLC, DAG-lipase, PKC or tyrosine kinase

In A549 cells permeabilized with 40 μg/mL saponin

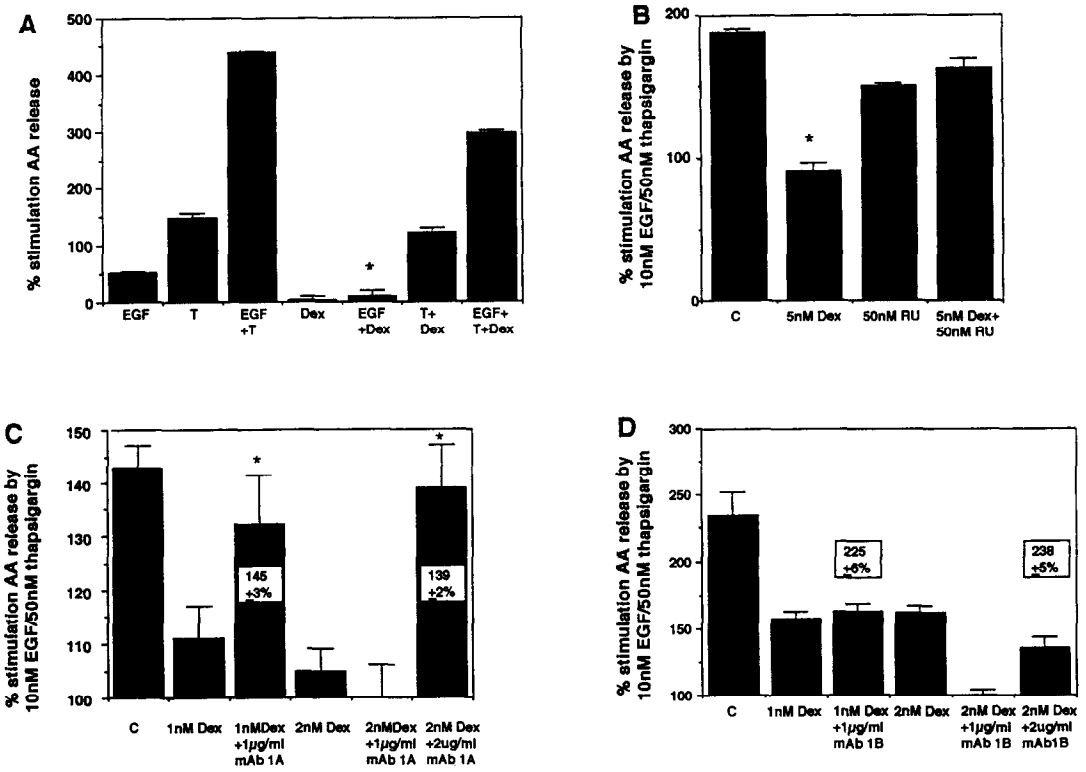

Fig. 2. Dexamethasone inhibits EGF but not thapsigargin stimulation of ^{3}H-AA via the induction of lipocortin-1, a GR-mediated event. A, A549 cells were treated with 5 nM dexamethasone for 3 hr prior to the addition of 10 nM EGF or 50 nM thapsigargin (T) or their combination (*$P < 0.001$ compared to EGF alone). B, Cells were treated for 3 hr with 5 nM dexamethasone, 50 nM RU486 (RU) or their combination prior to the addition of 10 nM EGF/50 nM thapsigargin (*$P < 0.001$ compared to all other treatments). C, Cells were treated for 3 hr with 1 or 2 nM dexamethasone with 1 or 2 μg/mL mAb 1A as indicated prior to the addition of 10 nM EGF/50 nM thapsigargin (*$P < 0.001$ compared to dexamethasone alone). Values in panel indicate effect of mAb alone. D, Cells were treated for 3 hr with 1 or 2 nM dexamethasone with 1 or 2 μg/mL mAb 1B prior to the addition of EGF/thapsigargin. Values in panel indicate effect of mAb alone. Each bar is the mean of 3 wells ± 1 SD.

for 3 min and then treated with neomycin for 3 hr prior to stimulation with EGF/thapsigargin, ^{3}H-AA release was not significantly affected (Fig. 4A). Similarly, in cells pre-treated for 3 hr with the DAG-lipase inhibitor RG80267, stimulation of ^{3}H-AA also remained unaffected (Fig. 4B). In A549 cells pre-treated for 3 hr with the PKC inhibitor H7 prior to stimulation with EGF/thapsigargin, ^{3}H-AA was not significantly affected (Fig. 4C). Identical results were obtained with the PKC inhibitor staurosporine (1 nM–10 μM, data not shown). The tyrosine kinase inhibitor methyl-cinnamate also failed to block EGF/thapsigargin stimulation of ^{3}H-AA release when pre-incubated for 3 hr (Fig. 4D). Identical negative results were obtained with the tyrosine kinase inhibitor genestein (1 nM–10 μM, data not shown).

N-terminal peptides from lipocortin-1 mimic dexamethasone in the suppression of EGF/thapsigargin stimulated arachidonic acid release

Pre-treatment for 3 hr with peptide Lc1–12 has no effect on EGF/thapsigargin stimulated ^{3}H-AA release (Fig. 5A). However, peptide Lc13–25 significantly inhibits stimulated release in a dose-dependent manner from 20 μg/mL (10.9 μM) and above ($P < 0.001$), whereas a scrambled sequence of Lc13–25 inhibited release to a lesser extent at 50 μg/mL and above (Fig. 5B). Peptide Lc21–33 also inhibits stimulated ^{3}H-AA release from 20 μg/mL (13.1 μM) and above but to a lesser degree (Fig. 5A), concentrations of 2 and 10 μg/mL were weakly stimulatory. This confirms our previous observations that Lc13–25 is the most active sequence from the N-terminal portion of lipocortin-1 in suppression of PGE_2 release and proliferation of A549 cells [2]. The peptide Lc13–25(Tyr-Me^{21}) is methylated on tyrosine 21 and cannot be phosphorylated. This peptide inhibited the EGF/thapsigargin stimulation of ^{3}H-AA release to a much lesser degree than Lc13–25 (Fig. 5C), thus suggesting that the availability of Tyr^{21} for phosphorylation is important for activity. Pre-treatment with 20 μg/mL Lc13–25 for 3 hr completely inhibits the stimulation of ^{3}H-AA release by 10 nM EGF whereas thapsigargin stimulated release is inhibited but to a lesser degree (Fig. 5D). Therefore, the inhibition of EGF/thapsigargin stimulated ^{3}H-AA release is predominantly at the level of the EGF pathway.

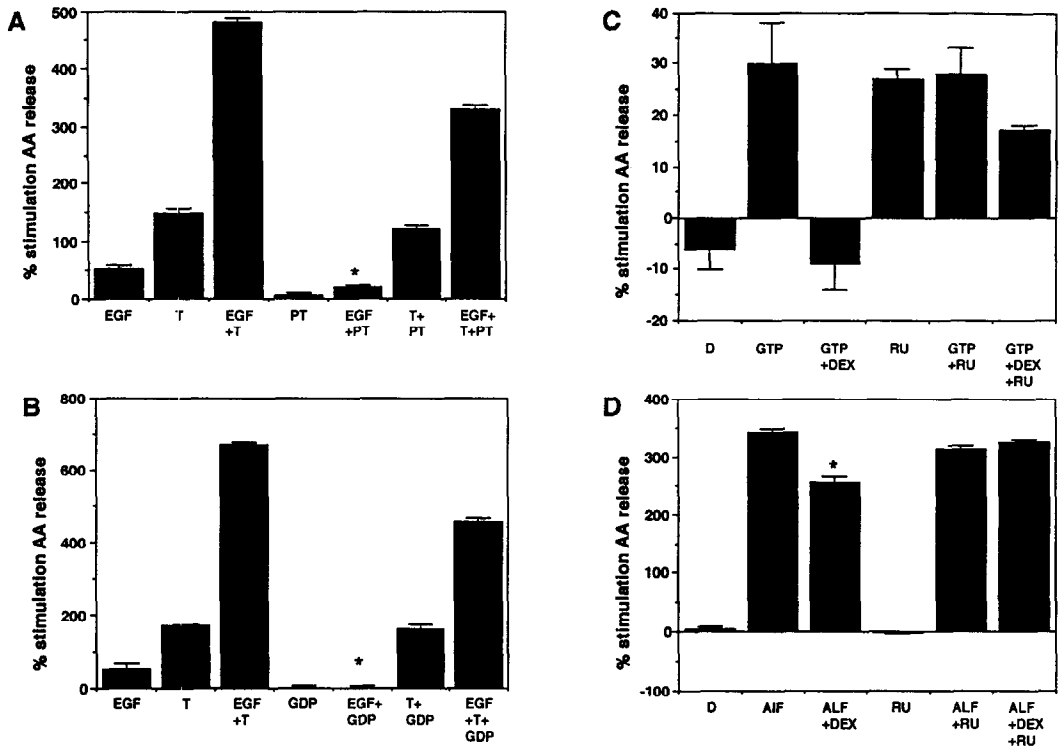

Fig. 3. Arachidonic acid release is mediated by G protein activation. A, A549 cells were pre-treated with 10 ng/mL pertussis toxin for 3 hr prior to the addition of 10 nM EGF, 50 nM thapsigargin (T) or their combination. B, Saponin permeabilized cells were pre-treated with 100 μM GDPβS for 30 min prior to the addition of EGF/thapsigargin. C, Cells were pre-treated with 5 pM dexamethasone (D), 50 pM RU486 (RU) or their combination for 3 hr. Then following saponin permeabilization 100 μM GTPγS was added for a further 30 min. D, Cells were pre-treated with 5 nM dexamethasone (D), 50 nM RU486 (RU) or their combination for 3 hr prior to the addition of 25 nM NaF and 10 μM $AlCl_3$ (AlF) for 1 hr. Each bar is the mean of 3 wells $\pm$ 1 SD.

G protein-dependent activation of arachidonic acid release is inhibited by glucocorticoids via the induction of lipocortin-1

The stimulation of ^{3}H-AA release by 100 μM GTPγS is completely inhibited by pre-treatment for 3 hr with 2 pM dexamethasone. This effect is completely reversed by co-incubation with 1 μg/mL of the neutralizing mAb 1A but not by the non-neutralizing mAb 1B (Fig. 6A). Pre-treatment of GTPγS stimulated cells with peptide Lc13–25 for 3 hr inhibits the release of ^{3}H-AA in a dose-dependent manner (0.1–2 μg/mL) whereas the scrambled peptide did not inhibit (Fig. 6B).

DISCUSSION

In this paper we report that EGF and thapsigargin each stimulate an increase in the release of ^{3}H-AA from A549 cells between approximately 70–120-fold. However, combined use of the two agents results in a dose-dependent, synergistic stimulation to a maximum of between approximately 400 and 700% above control levels. The activity of $cPLA_2$ in U937 cells [6, 7, 14] and also in A549 cells [8, 10] has been shown to rise following physiological increases in Ca_i^{2+}, a phenomenon that is mimicked by treatment with thapsigargin [15, 16]. This is thought to be achieved by a translocation of $cPLA_2$ from the cytosol to membrane where the phospholipid substrate is localized [14]. Our data is consistent with this hypothesis but suggests that EGF is able to further stimulate $cPLA_2$ activity presumably by a stable modification as has been described previously [17]. Therefore, it seems likely that $cPLA_2$ activity is under a dual control of Ca_i^{2+} and growth factor-dependent activation.

Activation of ^{3}H-AA release by EGF has previously been demonstrated to be pertussis toxin sensitive [11, 12]. Pertussis toxin is a power inhibitor of G proteins belonging to the G_i and G_o families. We have confirmed that EGF stimulation of ^{3}H-AA is also inhibited by pertussis toxin in A549 cells, whereas thapsigargin stimulation of ^{3}H-AA release is unaffected by this treatment. Furthermore, treatment of saponin permeabilized A549 cells with GDPβS completely inhibited EGF stimulation of ^{3}H-AA release, whereas thapsigargin-stimulated release was not significantly affected. Therefore, these results indicate that the dual control of $cPLA_2$ in A549 cells by EGF/thapsigargin appears to be mediated by a G protein-dependent mechanism activated through the EGF signalling pathway.

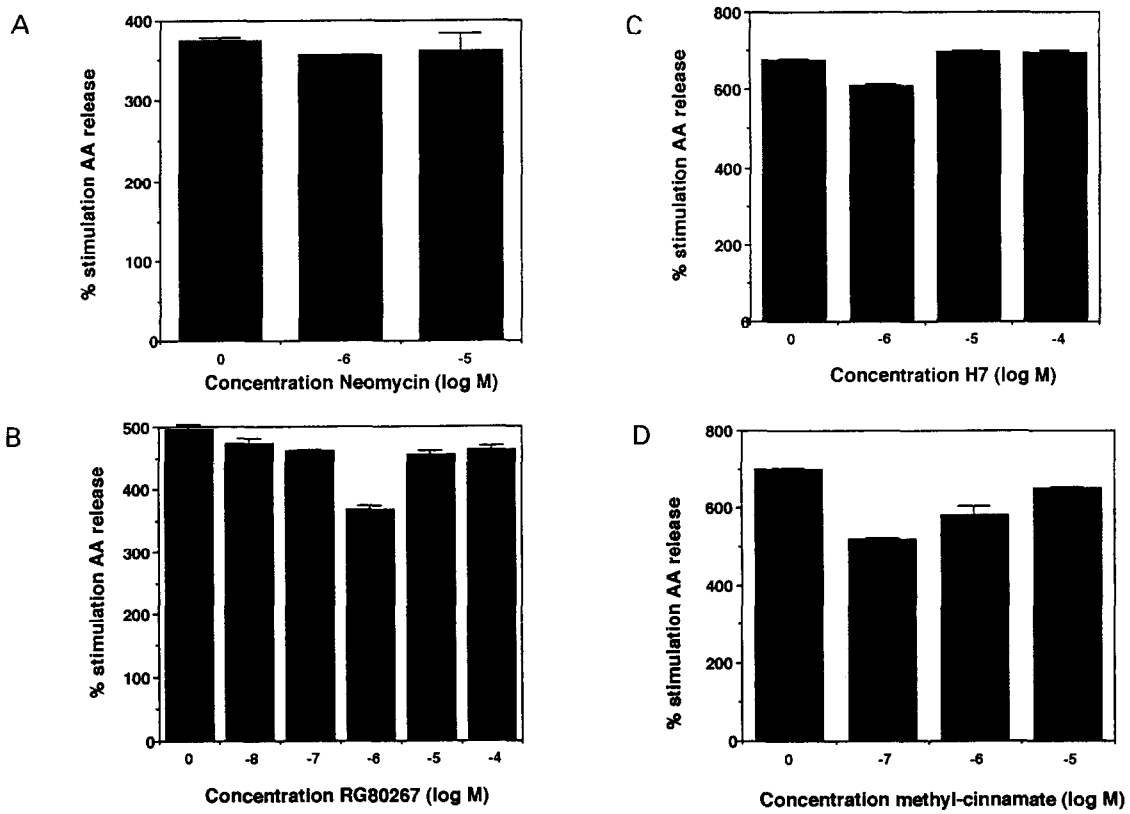

Fig. 4. Inhibitors of PLC, PKC, DAG-lipase and tyrosine kinase do not inhibit ^{3}H-AA release. A, Saponin permeabilized A549 cells were pre-treated with neomycin for 3 hr prior to the addition of 10 nM EGF/50 nM thapsigargin. B, C, D, All cultures were pre-treated with inhibitor for 3 hr prior to the addition of EGF/thapsigargin. Each bar is the mean of 3 wells ± 1 SD.

Extracellular Ca^{2+} appears to be unnecessary for the immediate EGF stimulation of PGE_2 release [12] and agonist activation of ^{3}H-AA release in A549 cells [18] as both effects occur in EDTA-containing media. We show here that the stimulatory effect of thapsigargin on ^{3}H-AA release which is maximal after 30 min is completely lost by 120 min presumably as Ca_i^{2+} stores are discharged (Fig. 1C). This pool can ultimately be replenished only from the extracellular medium. Influx of Ca^{2+} can itself be stimulated by EGF in a G protein-mediated manner but by a process which is insensitive to pertussis toxin, suggesting that G_i and G_o proteins are not involved [19]. However, this effect of EGF is mimicked by cholera toxin which activates G_s and AlF_4^- which is a universal hetero-trimeric G protein activator [19] suggesting that other members of the G protein family are involved in the Ca^{2+} influx. As pertussis toxin does not inhibit EGF stimulation of Ca^{2+} influx [19] then the inhibition of EGF stimulated ^{3}H-AA release we describe here is presumably only manifested at the level of the signal transduction cascade that activates $cPLA_2$. Clearly the integration of G protein-dependent Ca^{2+} signalling in mediating the effects of growth factors needs to be investigated further.

G protein-dependent activation of ^{3}H-AA release is well documented and our results now further extend this concept to A549 cells. Treatment of saponin permeabilized cells with 100 μM GTPγS stimulates ^{3}H-AA release by approximately 30%. Furthermore, treatment with AlF_4^- for 1 hr stimulates ^{3}H-AA release by over 300% above control levels. AlF_4^- activates hetero-trimeric G proteins non-specifically and particularly the G protein-dependent Ca^{2+} influx [19]. This would account for the much greater stimulation of ^{3}H-AA as $cPLA_2$ activity is increased by a combined rise in Ca_i^{2+} and G protein-dependent activation. Cholera toxin treatment had no effect on ^{3}H-AA release in these cells (data not shown).

Our previous work has suggested that PLC does not make a major contribution to ^{3}H-AA release in A549 cells [18] and the data presented here further supports that. Rather the role of $cPLA_2$ seems to be more important. The mechanism of activation of $cPLA_2$ by the tyrosine kinase activity of the EGF receptor has been unclear as $cPLA_2$ appears mainly to be phosphorylated on Ser-505 [20]. The discovery that $cPLA_2$ could be phosphorylated and activated by the MAPK [21] otherwise known as ERK and also by protein kinase C [22] shed some light on this problem. Adrenergic stimulation of fibroblasts resulted in activation of $p21^{ras}$ and a rapid phosphorylation of MAPK which increased the catalytic activity [23], both effects were blocked by

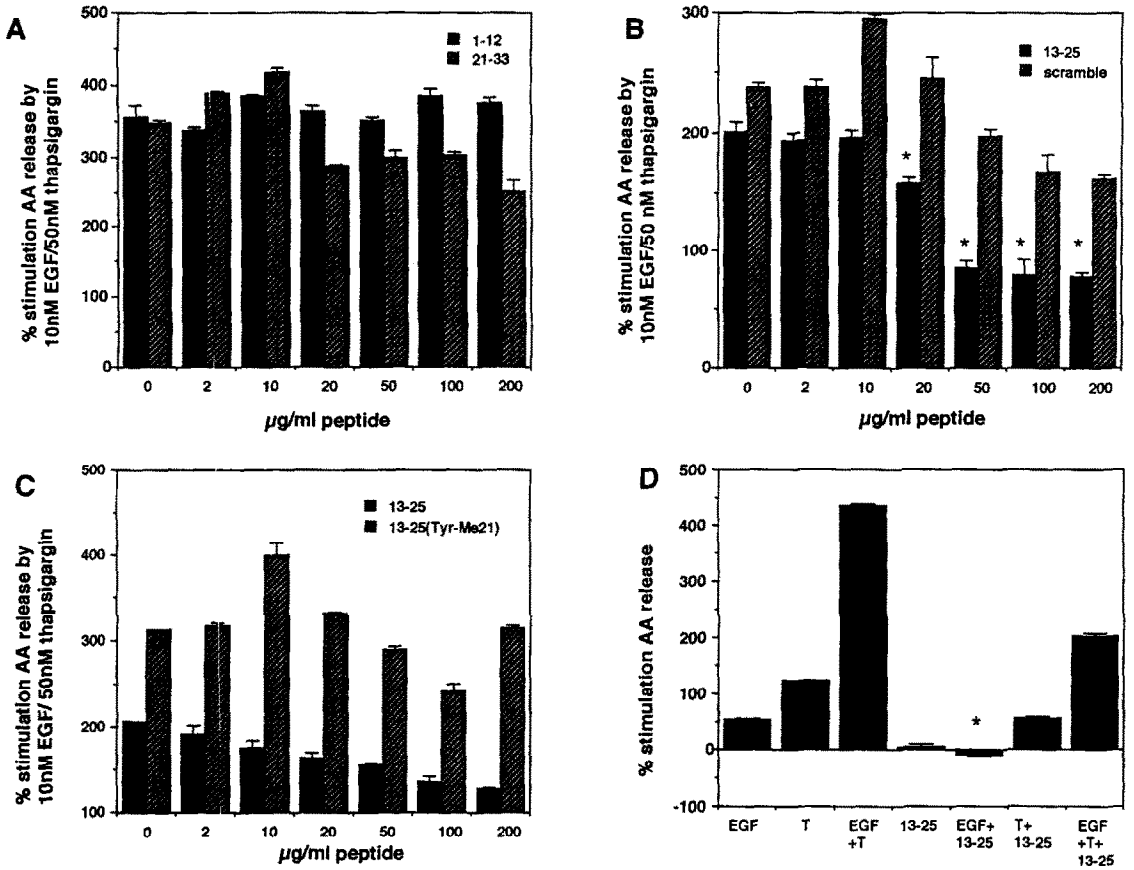

Fig. 5. N-terminal peptides from lipocortin-1 inhibit EGF/thapsigargin stimulation of ^{3}H-AA. A, A549 cells were pre-treated with peptides Lc1–12 or Lc21–33 for 3 hr prior to the addition of 10 nM EGF/ 50 nM thapsigargin. B, Cells were pre-treated with peptides Lc13–25 or its scrambled alternative for 3 hr prior to the addition of EGF/thapsigargin. C, Cells were pre-treated with peptide Lc13–25(Tyr-Me^{21}) for 3 hr prior to the addition of EGF/thapsigargin. D, Cells were pre-treated with 20 μg/mL Lc13–25 for 3 hr prior to the addition of 10 nM EGF, 50 nM thapsigargin or their combination (*$P < 0.001$ compared to EGF alone). Each bar is the mean of 3 wells ± 1 SD.

pertussis toxin [23]. MAPK can act as a substrate for diverse enzymes including tyrosine, serine and threonine kinases [24] and thus could play a pivotal role in coordinating cell signalling. Recently, the direct activation of MAPK in cells transfected with $p21^{ras}$ has been demonstrated circumventing the requirement for growth factor activation [25]. This reinforces the concept that the cascade of events which follows growth factor treatment is an activation of $p21^{ras}$ which in turn activates MAPK and subsequently $cPLA_2$. Other intermediary kinases of other pathways may be involved [26, 27] and the integration of these pathways is currently being evaluated [28, 29]. In particular, however, our results suggest that PKC does not appear to be involved in this case.

The EGF/thapsigargin stimulation of ^{3}H-AA release is significantly inhibited by pre-treatment with dexamethasone in a dose-dependent manner from concentrations of 1 pM and above. This reflects the sensitivity of A549 cell proliferation to inhibition by glucocorticoids [1]. Dexamethasone treatment significantly inhibits EGF stimulation of ^{3}H-AA release whereas thapsigargin stimulation is unaffected. Furthermore, the effect of dexamethasone is significantly reversed by a 10-fold excess of RU486. This indicates that glucocorticoids can inhibit the EGF stimulated pathway of $cPLA_2$ activation by a receptor mediated event. The stimulation of ^{3}H-AA release by GTPγS is also completely inhibited by pre-treatment with dexamethasone and this effect is also reversed by a 10-fold excess of RU486. This indicates that the glucocorticoid suppression of this pathway is downstream of G protein activation. Similarly, AlF_4-stimulation of ^{3}H-AA release is also significantly inhibited by dexamethasone but to a lesser degree indicating that a component of AlF_4-activation is insensitive to glucocorticoids. However, the inhibition by dexamethasone is completely reversed by RU486 reinforcing the concept that glucocorticoids inhibit G protein activation of ^{3}H-AA by occupancy of their receptor.

We have previously described how treatment of A549 cells with dexamethasone results in the appearance of lipocortin-1 on the surface of these

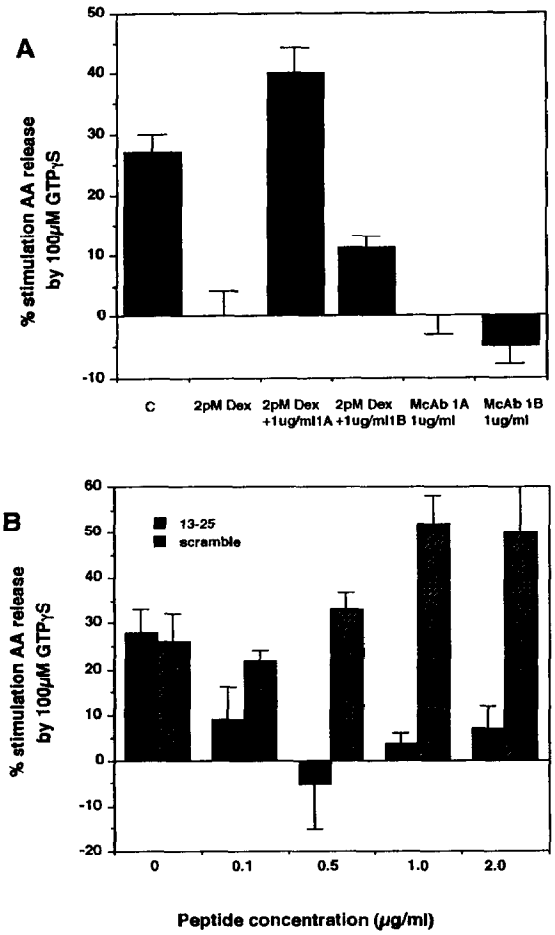

Fig. 6. Dexamethasone inhibits G protein-dependent activation of ^{3}H-AA release via the induction of lipocortin-1. A, A549 cells were pre-treated with 2 pM dexamethasone and with 1 μg/mL mAb 1A or 1B for 3 hr. Then following saponin permeabilization, treated with 100 μM GTPγS for 30 min (*$P < 0.001$, compared to dexamethasone alone). B, Cells were pre-treated for 3 hr with peptide prior to saponin permeabilization followed by treatment with 100 μM GTPγS for 30 min. Each bar is the mean of 3 wells ± 1 SD.

cells [1]. Furthermore, by using specific neutralizing monoclonal antibodies (mAb 1A) and anti-sense oligonucleotides we have shown that lipocortin-1 mediates the inhibition of cell growth induced by dexamethasone by suppressing the release of PGE_2 necessary for their proliferation [1, 30]. The addition of recombinant human lipocortin-1 to A549 cells suppresses PGE_2 release and cell growth [1]. We now show here that the inhibition of EGF/thapsigargin stimulated ^{3}H-AA release by dexamethasone is reversed by co-incubation with the neutralizing monoclonal antibody 1A, whereas the non-neutralizing antibody 1B had little effect. This indicates that lipocortin-1 inhibits the release of PGE_2 and A549 cell growth by directly blocking the stimulation of the release of ^{3}H-AA. Moreover, we have further shown that the dexamethasone inhibition of G protein-dependent activation of ^{3}H-AA release is also reversed by anti-lipocortin-1 mAb 1A and not 1B indicating that the action of lipocortin-1 is downstream of G protein activation.

Recombinant lipocortin-1 mimics the effects of glucocorticoids in inhibiting inflammation [31] and cell growth [1]. Re-folding of the recombinant protein is essential for activity [32], which is as a consequence highly labile. However, it is now apparent that the full length protein is not essential for biological activity as a fragment 1–188 suppresses cytokine-induced fever [33] and ischaemic brain damage in the rat [34]. Furthermore, a peptide fragment 2–22 from the N-terminus of lipocortin-1 still retains anti-inflammatory activity [35].

We report here that peptides from the N-terminus of lipocortin-1 block both EGF and G protein-dependent activation of ^{3}H-AA release. Lc13–25 is the most active sequence, Lc21–33 less so, and peptides Lc1–12 and Lc13–25(Tyr-Me^{21}) are almost inactive which is consistent with the profile of activity for growth and PGE_2 suppression that we have reported previously [2]. These results indicate that the core sequence of activity resides around the Tyr 21 residue, which is itself essential for activity, suggesting that the peptides could inhibit tyrosine kinase activity. Lipocortin-1 is apparently a substrate for the tyrosine kinase activity of the EGF and insulin receptor [36, 37], however, as these studies employed partially purified preparations of receptor the possibility that other intermediary kinases are involved cannot be excluded. Our data clearly shows that both dexamethasone and peptide Lc13–25 inhibit the G protein-dependent activation of ^{3}H-AA release suggesting that lipocortin-1 is not a direct substrate for growth factor receptors but inhibits the signal transduction cascade further downstream. Since MAPK phosphorylates $cPLA_2$ on a serine residue [20] it is unlikely that the peptide Lc13–25 inhibits at this step. Rather, our data indicates the likely site of action is between G protein and MAP kinase activation (Fig. 7).

Our previous work has highlighted the importance of the cell surface expression of lipocortin-1—the pool which is induced following glucocorticoid treatment [1]. Furthermore, the exogenous application of recombinant lipocortin-1 or neutralizing antibodies is highly effective in cell culture and presumably these effects are also manifest at the cell surface [38]. The existence of specific saturable binding sites for lipocortin-1 has been described on the surface of human and murine leukocytes [39] and also in A549 cells (Goulding, pers. commun.) which could help to explain the importance of the cell surface form of lipocortin-1. The properties of the N-terminal fragments of lipocortin-1 could also be mediated by this cell surface binding protein. The Me-Tyr^{21} derivative was much less effective in inhibiting stimulated ^{3}H-AA release suggesting that the availability of the tyrosine residue for phosphorylation or binding is important for activity. However, we cannot exclude the possibility that this derivative peptide is sufficiently changed in confirmation to preclude interaction with the binding protein. Indeed, some of the weak stimulatory

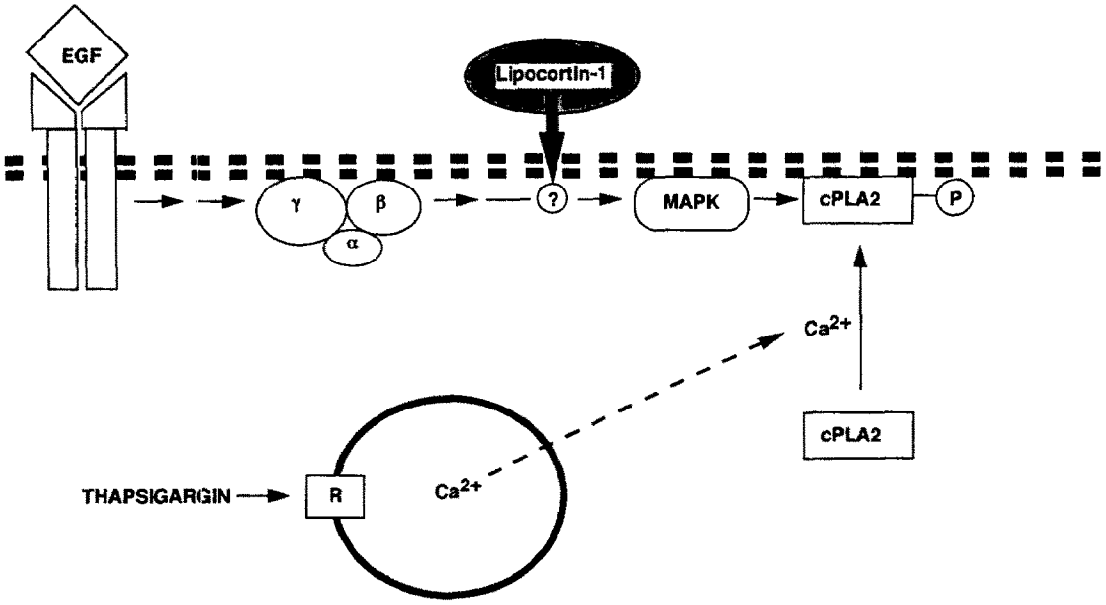

Fig. 7. Lipocortin-1 inhibits EGF activation of $cPLA_2$. An increase in Ca_i^{2+} results in a translocation of $cPLA_2$ to the plasma membrane, an effect which is insensitive to glucocorticoids or lipocortin-1. Activation of EGF receptor results in phosphorylation and activation of $cPLA_2$, an effect that is mediated by G protein and MAPK activation. This pathway is sensitive to glucocorticoids and lipocortin-1.

effects of this peptide and others we have shown here could be accounted for by such binding phenomena.

The results presented here provide further evidence for the role of lipocortin-1 in mediating the effects of glucocorticoids in suppressing arachidonic acid release. Furthermore, this property is retained by N-terminal peptide fragments of the native protein which do not have the capacity to sequester phospholipid. Moreover, we have identified a mechanism whereby glucocorticoids inhibit G protein-mediated cell signalling.

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REFERENCES

1. Croxtall JD and Flower RJ, Lipocortin 1 mediates dexamethasone-induced growth arrest of the A549 lung adenocarcinoma cell line. *Proc Natl Acad Sci USA* **89**: 3571–3575, 1992.
2. Croxtall JD, Waheed S, Choudhury Q, Anand R and Flower RJ, N-terminal peptide fragments of lipocortin-1 inhibit A549 cell growth and block EGF-induced stimulation of proliferation. *Int J Cancer* **54**: 153–158, 1993.
3. Handler JA, Danilowicz RM and Eling TE, Mitogenic signaling by epidermal growth factor (EGF), but not platelet-derived growth factor, requires arachidonic acid metabolism in BALB/c3T3 cells. *J Biol Chem* **265**: 3669–3673, 1990.
4. Blackwell GJ, Carnuccio R, Di Rosa M, Flower RJ, Parente L and Persico P, Macrocortin: a polypeptide causing the anti-phospholipase effect of glucocorticoids. *Nature* **287**: 147–149, 1980.
5. Diez E and Mong S, Purification of a phospholipase A_2 from human monocytic leukemic U937 cells. *J Biol Chem* **265**: 14654–14661, 1990.
6. Clark JD, Milona N and Knopf JL, Purification of a 110-kilodalton cytosolic phospholipase A_2 from the human monocytic cell line U937. *Proc Natl Acad Sci USA* **87**: 7708–7712, 1990.
7. Kramer RM, Roberts EF, Manetta J and Putnam JE. The Ca^{2+}-sensitive cytosolic phospholipase A_2 is a 100-kDa protein in human monoblast U937 cells. *J Biol Chem* **266**: 5268–5272, 1991.
8. Tokumoto H, Croxtall JD, Choudhury Q and Flower RJ, Phospholipase A_2-induced stimulation of A549 lung adenocarcinoma cell line proliferation. *Biochim Biophys Acta* **1164**: 236–242, 1993.
9. Clark S and Dunlop M, Modulation of phospholipase A_2 activity by epidermal growth factor (EGF) in CHO cells transfected with EGF receptor. *Biochem J* **274**: 715–721, 1991.
10. Neagos GR, Feyssa A and Peters-Golden M, Phospholipase A_2 in alveolar type II cells: biochemical and immunologic characterization. *Am J Physiol* **264**: L261–L268, 1993.
11. Burch RM, Luini A and Axelrod J, Phospholipase A_2 and phospholipase C are activated by distinct GTP-binding proteins in response to α_1-adrenergic stimulation in FRTL5 thyroid cells. *Proc Natl Acad Sci USA* **83**: 7201–7205, 1986.
12. Teitelbaum I, The epidermal growth factor receptor is coupled to a phospholipase A_2-specific pertussis toxin-inhibitable guanine nucleotide-binding regulatory protein in cultured rat inner medullary collecting tubule cells. *J Biol Chem* **265**: 4218–4222, 1990.

13. Liu P, Wen M and Hayashi J, Guanine nucleotide-binding protein stimulates arachidonic acid metabolism in TEA3A1 thymic epithelial cells by stimulating release and inhibiting incorporation of arachidonic acid. *Biochem Molec Biol Int* **31**: 613–625, 1993.
14. Clark JD, Lin L-L, Kriz RW, Ramesha CS, Sultzman LA, Lin AY, Milona N and Knopf JL, A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca^{2+}-dependent translocation domain with homology to PKC and GAP. *Cell* **65**: 1043–1051, 1991.
15. Thastrup O, Dawson AP, Scharff O, Foder B, Cullen PJ, Drobak BK, Bjerrum PJ, Christensen SB and Hanley MR, Thapsigargin, a novel molecular probe for studying intracellular calcium release and storage. *Agents Actions* **27**: 17–23, 1989.
16. Himpens B, De Smedt H and Casteels R, Intracellular Ca^{2+} signaling induced by vasopressin, ATP, and epidermal growth factor in epithelial LLC-PK_1 cells. *Am J Physiol* **265**: C966–C975, 1993.
17. Spaargaren M, Wissink S , Defize LHK, De Laat SW and Boonstra J, Characterization and identification of an epidermal-growth-factor-activated phospholipase A_2. *Biochem J* **287**: 37–43, 1992.
18. Tokumoto H, Croxtall JD and Flower RJ, Differential role of extra- and intracellular calcium in bradykinin and interleukin 1α stimulation of arachidonic acid release from A549 cells. *Biochim Biophys Acta* **1211**: 301–309, 1994.
19. Kuryshev YA, Naumov AP, Avdonin PV and Mozhayeva GN, Evidence for involvement of a GTP-binding protein in activation of Ca^{2+} influx by epidermal growth factor in A431 cells: effects of fluoride and bacterial toxins. *Cellular Signalling* **5**: 555–564, 1993.
20. Lin L-L, Lin AY and Knopf JL, Cytosolic phospholipase A_2 is coupled to hormonally regulated release of arachidonic acid. *Proc Natl Acad Sci USA* **89**: 6147–6151, 1992.
21. Lin L-L, Wartmann M, Lin AY, Knopf JL, Seth A and Davis RJ, $cPLA_2$ is phosphorylated and activated by MAP kinase. *Cell* **72**: 269–278, 1993.
22. Nemenoff RA, Winitz S, Qian N-X, Van Putten V, Johnson GL and Heasley LE, Phosphorylation and activation of a high molecular weight form of phospholipase A_2 by p42 microtubule-associated protein 2 kinase and protein kinase C. *J Biol Chem* **268**: 1960–1964, 1993.
23. Alblas J, van Corven EJ, Hordijk PL, Milligan G and Moolenaar WH, G_i-mediated activation of the $p21^{ras}$-mitogen-activated protein kinase pathway by α_2-adrenergic receptors expressed in fibroblasts. *J Biol Chem* **268**: 22235–22238, 1993.
24. Her J-H, Lakhani S, Zu L, Vila J, Dent P, Sturgill TW and Weber MJ, Dual phosphorylation and autophosphorylation in mitogen-activated protein (MAP) kinase activation. *Biochem J* **269**: 25–31, 1993.
25. de Vries-Smits AMM, Burgering BMTh, Leevers SJ, Marshall CJ and Bos JL, Involvement of $p21^{ras}$ in activation of extracellular signal-regulated kinase 2. *Nature* **357**: 602–604, 1992.
26. Ahn NG, Seger R, Bratlien RL, Diltz CD, Tonks NK and Krebs EG, Multiple components in an epidermal growth factor-stimulated protein kinase cascade. *J Biol Chem* **266**: 4220–4227, 1991.
27. Lange-Carter CA, Pleiman CM, Gardner AM, Blumer KJ and Johnson GL, A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. *Science* **260**: 315–319, 1993.
28. Ahn NH, The MAP kinase cascade. Discovery of a new signal transduction pathway. *Molec Cell Biochem* **127/128**: 201–209, 1993.
29. Chao T-SO, Foster DA, Rapp UR and Rosner MR, Differential Raf requirement for activation of mitogen-activated protein kinase by growth factors, phorbol esters, and calcium. *J Biol Chem* **269**: 7337–7341, 1994.
30. Croxtall JD and Flower RJ, Antisense oligonucleotides to human lipocortin-1 inhibit glucocorticoid-induced inhibition of A549 cell growth and eicosanoid release. *Biochem Pharmacol* **48**: 1729–1734, 1994.
31. Cirino G, Peers, SH, Flower RJ, Browning JL and Pepinsky RB, Human recombinant lipocortin 1 has acute local anti-inflammatory properties in the rat paw edema test. *Proc Natl Acad Sci USA* **86**: 3428–3432, 1989.
32. Browning JL, Ward MP, Wallner BP and Pepinsky RB, Studies on the structural properties of lipocortin-1 and the regulation of its synthesis by steroids. In: *Cytokines and Lipocortins in Inflammation and Differentiation* (eds. Melli M and Parente L), pp. 27–45. Wiley-Liss, New York, 1990.
33. Carey F, Forder R, Edge MD, Greene AR, Horan MA, Strijbos PJLM and Rothwell NJ, Lipocortin-1 modifies the pyrogenic actions of cytokines in the rat. *Am J Physiol* **259**: R266–R269, 1990.
34. Relton JK, Strijbos PJLM, O'Shaughnessy CT, Carey F, Forder RA, Tilders FJH and Rothwell NJ, Lipocortin-1 is an endogenous inhibitor of ischemic damage in the rat brain. *J Exper Med* **174**: 305–310, 1991.
35. Cirino G, Cicala C, Sorrentino L, Ciliberto G, Arpaia G, Perretti M and Flower RJ, Anti-inflammatory actions of an N-terminal peptide from human lipocortin 1. *Br J Pharmacol* **108**: 573–574, 1993.
36. Pepinsky RB and Sinclair LK, Epidermal growth factor-dependent phosphorylation of lipocortin. *Nature* **321**: 81–85, 1986.
37. Karasik A, Pepinsky RB, Shoelson SE and Kahn CR, Lipocortins 1 and 2 as substrates for the insulin receptor kinase in rat liver. *J Biol Chem* **263**: 11862–11867, 1988.
38. Pepinsky RB, Sinclair LK, Dougas I, Liang CM, Lawton P and Browning JL, Monoclonal antibodies to lipocortin-1 as probes for biological function. *FEBS* **261**: 247–252, 1990.
39. Goulding NJ, Luying P and Guyre PM, Characteristics of lipocortin 1 binding to the surface of human peripheral blood leukocytes. *Biochem Soc Trans* **18**: 1237–1238, 1990.