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Modulation of phospholipase A_2 activity by epidermal growth factor (EGF) in CHO cells transfected with human EGF receptor

Role of receptor cytoplasmic subdomain

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Activation of phospholipase A_2 (PLA_2) in response to external stimuli may play a pivotal role in signal-transduction pathways via the generation of important cellular intermediates, including prostaglandins. Epidermal growth factor (EGF) has been shown to modulate prostaglandin production, possibly via direct activation of PLA_2 or indirectly via interaction with a PLA_2-modifying protein such as lipocortin I. We have investigated these pathways with two CHO cell-lines, one (CHOwt) transfected with the full-length human EGF receptor and the second (CHO 11) with a deletion mutant, Δ990, that has lost the autophosphorylation sites and part of the internalization domain. CHOwt cells responded to EGF with a rapid rise in lysophosphatidylcholine and arachidonic acid release concomitant with an increase in prostaglandin production. However, in the non-internalizing CHO 11 cells no such activation of PLA_2 was observed. This was not due to an intrinsic lack of PLA_2 in these cells, as PLA_2 activation was shown on melittin addition, nor was this difference due to a defect in intracellular pathways, as arachidonic acid was released from both cell types by Ca^{2+} and protein kinase C modulators. However, only in CHOwt cells were these responses potentiated by concomitant addition of EGF. Thus the cytoplasmic subdomain of the EGF receptor, containing the major sites of autophosphorylation and the internalization domain, seems to be involved in the activation of PLA_2 by EGF. In addition, we have shown that phosphorylation of lipocortin I is unlikely to play a role in PLA_2 activation. In CHOwt cells and a positive control cell line, A431, activation of PLA_2 was complete by 10 min, at which time there was no evidence of lipocortin I phosphorylation.

INTRODUCTION

In a number of cell types epidermal growth factor (EGF) is shown to modulate prostaglandin production. These cells include dog kidney (Levine & Hassid, 1977), mouse fibrosarcoma (Hirata *et al.*, 1984), neonatal-rat calvaria (Tashjian & Levine, 1978), and fetal long bones (Lorenzo *et al.*, 1986), rat keratinocytes (Aoyagi *et al.*, 1985), rat aortic smooth-muscle cells (Blay & Hollenberg, 1988), human amnion cells (Casey *et al.*, 1988), rat kidney mesangial cells (Margolis *et al.*, 1988*a*,*b*) and BALB/c 3T3 fibroblasts (Nolan *et al.*, 1988; Handler *et al.*, 1990). Investigations in the latter cell indicate that, after EGF stimulation, the release of arachidonic acid and its subsequent metabolism to prostaglandin is necessary for modulation of EGF-dependent c-*myc* expression and initiation of mitogenesis. In other cell types EGF-stimulated prostaglandin production is associated with either functional or proliferative changes. The rate-limiting step for synthesis of prostaglandins is believed to be the liberation of arachidonic acid from membrane phospholipids by the action of phospholipase A_2 (PLA_2) (Flower & Blackwell, 1976).

However, arachidonic acid may also be made available by a second major pathway. The sequential activation of phospholipase C (PLC) and diacylglycerol lipase may release arachidonic acid from diacylglycerols formed from arachidonate-enriched phosphatidylinositols. EGF activation of this pathway has been shown by the demonstration of increased inositol phosphates, indicating PLC action on phosphatidylinositols, including phosphatidylinositol 4,5-bisphosphate in Rat 1 cells (Muldoon *et al.*, 1988), WB (a non-transformed continuous rat liver epithelial cell line) (Hepler *et al.*, 1988), A431 (Moolenaar *et al.*, 1986; Wahl & Carpenter, 1988) and human hepatocellular carcinoma cell lines (PLC/PRF/5) over-expressing EGF receptors (EGF-R) (Gilligan *et al.*, 1988). It has been demonstrated recently that the tyrosine kinase activity of the receptor is essential for the association of an isoform of PLC ($PLC\gamma$) with the EGF-R in EGF-R-transfected NIH3T3 cells, possibly an initial event in the transduction process (Margolis *et al.*, 1990). However, despite the presence of the EGF-R in mesangial cells, no activation of PLC precedes prostaglandin production after EGF stimulation (Margolis *et al.*, 1988*a*,*b*), suggesting that PLA_2 activation alone may be involved in the EGF response. However, as shown in amnion cells PGH_2 synthetase induction follows prolonged (1 h) EGF stimulation, without an effect on PLA_2 activity, and could account for the elevated prostanoid production seen in these cells (Casey *et al.*, 1988). It has taken the recent demonstration by Boneventre *et al.* (1990) of EGF-enhanced soluble PLA_2 activity in mesangial cells to confirm a rapid release of arachidonic acid as a mechanism by which EGF increases prostaglandin production.

PLA_2 may be activated by several mechanisms. The Ca^{2+}-dependent binding of calmodulin to PLA_2 subunits induces activation (Moskowitz *et al.*, 1985), as does the presence of diacylglycerol, which does not alter the Ca^{2+} requirements (Burch, 1988). Additionally, activation of a guanine-nucleotide-binding protein, as shown by the action of guanosine 5′-[γ-thio]-

Abbreviations used: EGF, epidermal growth factor; EGF-R, EGF receptor; PLA_2, phospholipase A_2; PLC, phospholipase C; OAG, oleoylacetylglycerol; Cho, choline; PtdCho, phosphatidylcholine; *P*Cho, phosphocholine; Gro*P*Cho, glycerophosphocholine; CDPCho, CDP-choline; PGE, prostaglandin E; CHO, Chinese-hamster ovary; PCR, polymerase chain reaction; PKC, protein kinase C.

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triphosphate in saponin-permeabilized neutrophils (Nakashima *et al.*, 1988), independent of phosphoinositide-specific PLC activation, may activate PLA_2. Lastly, removal of the inhibitory action of two major PLA_2-inhibitor proteins (lipocortins I and II) by cellular regulatory mechanisms which involve phosphorylation reactions may also play a role in PLA_2 activation (Hirata, 1981).

One of these inhibitors of PLA_2, lipocortin I, has been shown to be a substrate for the EGF-R tyrosine kinase (Fava & Cohen, 1984; Pepinsky & Sinclair, 1986). Once phosphorylated, lipocortin I can no longer bind to and inhibit PLA_2 (Hirata, 1981). However, a causal link between EGF-R activation and stimulation of PLA_2 activity via phosphorylation of lipocortin I has not been demonstrated. Indeed, the time course used by other workers to demonstrate EGF-stimulated phosphorylation of lipocortin I is long (1 h), not consistent with the rapid activation of PLA_2 (Fava & Cohen, 1984; Pepinsky & Sinclair, 1986). The mechanisms involved in this potential regulation are unclear, but it has been proposed that internalization of the EGF-R may be required for phosphorylation of lipocortin I (Fava & Cohen, 1984).

To investigate the cellular events required for PLA_2 activation, we studied EGF stimulation of two transfected Chinese-hamster ovary (CHO) cell lines. CHOwt expresses the complete human EGF-R and responds normally to EGF (Chen *et al.*, 1987; Bertics *et al.*, 1988), whereas the CHO 11 cell line expresses a mutant EGF-R with a *C*-terminal deletion that removes the major autophosphorylation sites (Clark *et al.*, 1988). This mutant shows normal EGF binding, has EGF-stimulated kinase activity and responds mitogenically to EGF. The major alteration from normal EGF-R function is the inability of the mutant receptor to undergo EGF-induced receptor internalization. This cell line gives us the potential to look at the role of receptor internalization in the phosphorylation of lipocortin I. In addition, it was possible to examine the mechanism for EGF-stimulated prostaglandin production by investigation of EGF-stimulated phospholipid hydrolysis in cells labelled to equilibrium with [^{14}C]arachidonic acid, [^{14}C]stearic acid or [^{14}C]choline.

EXPERIMENTAL

Materials

Cells. CHO 11 cells (containing a mutant transfected EGF-R cDNA), derived as previously described (Clark *et al.*, 1988), were maintained in alpha medium (Gibco, Glen Waverly, Victoria, Australia) containing 10% (v/v) fetal-calf serum. The deletion in this EGF-R has now been definitively mapped to amino acid position 990. This was achieved by polymerase chain reaction (PCR) of first-strand cDNA derived from poly(A)$^+$ selected CHO 11 mRNA. Two rounds of PCR were used with two specific 5′ primers (nucleotides 3148–3167 and 3172–3189) and oligo(dT). A 130 bp fragment was amplified, and this was subcloned into M13 and sequenced. The EGF sequence ended at amino acid 990 and was followed by four unrelated amino acids before reaching a stop codon. CHO cells transfected with full-length EGF-R cDNA (CHOwt) were kindly provided by Dr. Gordon Gill (University of California, San Diego, CA, U.S.A.). These cells were maintained in alpha medium containing 10% dialysed fetal-calf serum and 5 μM-methotrexate (to maintain receptor expression levels via the *dhfr* gene in the original plasmid). The receptors in these cells have previously been characterized (Chen *et al.*, 1987; Bertics *et al.*, 1988). A431 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal-calf serum.

Chemicals. ^{125}I-EGF, [1-^{14}C]choline chloride and [5,6,8,11,12,14,15(n)-^{3}H]prostaglandin (PG) E_2 were purchased from Amersham International, Amersham, Bucks., U.K. Rabbit anti-PGE antibody was from Advanced Magnetics (Cambridge, MA, U.S.A.). Oleoylacetylglycerol (OAG), calcium ionophore A23187 (Calimycin), bee-venom melittin, which was further treated to remove residual PLA_2 contamination (Argiolas & Pisano, 1983) and all other chemicals were from Sigma (St. Louis, MO, U.S.A.). All solvents were from BDH, of AnalaR grade.

Unlabelled EGF was extracted and purified from male mouse submaxillary glands essentially by the method of Savage & Cohen (1972).

Methods

Internalization of ^{125}I-EGF. This was estimated by the acid-wash technique of Haigler *et al.* (1979). Cells (CHO 11 and CHOwt) were plated in 35 mm-diam. dishes at 1×10^6/ml. The cells were incubated at 37 °C in binding buffer [Earle's buffered salt solution/25 mM-Hepes (pH 7.4)/0.1% BSA] with 5×10^5 c.p.m. (50 ng) of ^{125}I-EGF with or without a 100-fold excess of unlabelled EGF in a final volume of 1 ml. At various times dishes were placed on ice and washed twice with ice-cold phosphate-buffered saline (161 mM-NaCl/1.6 mM-Na_2HPO_4/4 mM-NaH_2PO_4). Cells were incubated in acid-wash buffer (0.2 M-acetic acid/0.5 M-NaCl) for 6 min at 4 °C to remove surface-bound ^{125}I-EGF, and washed once in this buffer. To measure the internalized ^{125}I-EGF, the cells were lysed in 0.1 M-NaOH/2% (w/v) Na_2CO_3. The radioactivity in both the lysate and the acid wash was counted in an autogamma spectrometer.

Lipocortin I phosphorylation. For this, 10 cm dishes of confluent CHOwt, CHO 11 and A431 cells were washed once with serum-free medium and incubated for either 10 or 60 min in serum-free medium plus 1% BSA with or without 50 ng of EGF/ml. A crude membrane preparation was made by hypo-osmotic lysis of the cells, followed by differential centrifugation as described by Pepinsky & Sinclair (1986), with all buffers containing 1 mM-$CaCl_2$. The membranes were resuspended in phosphorylation buffer (20 mM-Hepes, 2 mM-$MgCl_2$, 1 mM-$CaCl_2$, 10 μM-Na_3VO_4), and 20 μl samples were incubated on ice for 10 min with 0.5 μCi of [γ-^{32}P]ATP. The reaction was stopped by the addition of 2 × SDS/PAGE sample buffer, and the samples were subjected to SDS/PAGE (10% acrylamide) under reducing conditions. Gels were then dried and autoradiographed with Hyperfilm-MP (Amersham).

Lipocortin I immunoblotting. A 20 μl sample of the membrane preparation from CHOwt and CHO 11 cells (prepared as described above) was subjected to SDS/PAGE (10% acrylamide) under reducing conditions. The membrane proteins were transferred to nitrocellulose and probed with anti-lipocortin I antibody (α-646; kindly provided by Dr. Blake Pepinsky, Biogen Research Corp., Cambridge MA, U.S.A.) at a dilution of 1:2000. The antigen–antibody complexes were detected with 5 μCi of ^{125}I-labelled Protein A.

Labelling of cells. Before confluency, cultures of CHOwt and CHO 11 cells were supplemented with [^{14}C]choline (Cho) (final radioactivity 1 μCi/ml), [1-^{14}C]arachidonic acid (50 nCi/ml) or [1-^{14}C]stearic acid (200 nCi/ml) and the cultures continued for 18 h. Cells were washed with alpha medium containing glucose (5.6 mM) and unlabelled Cho (25 μM), before preincubation for 20 min in alpha medium containing unlabelled Cho (25 μM) and arachidonic acid (3 μM). Cells were then incubated with various additions as described in the Results section for 10 min at 37 °C. Medium was removed rapidly and the cells were stored on ice after addition of 1 ml of ice-cold methanol.

Extraction and analysis of neutral lipids, phospholipids and choline metabolites. Cells in methanol were scraped and extracted into either chloroform/methanol (2:1, v/v) for Cho-labelled cells or chloroform/methanol/13 M-HCl (200:100:1, by vol.) for arachidonic acid-labelled cells. Water-soluble products were separated after addition of 0.75 ml of water or 2 M-KCl containing 50 mM-EDTA to the respective extractions. Medium was extracted in a similar manner. Lipid and water-soluble compounds were quantified by using the following systems for t.l.c.

Cho and stearic acid- and arachidonic acid-labelled phospholipids were separated by t.l.c. on activated borate-loaded plates (2.3% ethanolic borate) by using chloroform/methanol/water/saturated ammonia (120:75:6:4, by vol.) under saturated conditions. Neutral lipids were separated in a solvent system of light petroleum (b.p. 60–80 °C)/diethyl ether/acetic acid (70:30:1, by vol.). Phosphatidylcholine (PtdCho), phosphatidic acid, the combined phosphoinositides, diacylglycerol and non-esterified fatty acid were identified by co-localization with authentic standards.

Water-soluble choline metabolites in medium or aqueous cellular extracts were separated by t.l.c. on laned silica gel G plates by using methanol/0.5% NaCl/saturated ammonia (50:50:1, by vol.) under unsaturated conditions. Silica-gel areas containing metabolites, identified after autoradiography, were removed, added to water (500 μl, pH 4) and quantified by liquid-scintillation spectrometry. The identities of Cho and its metabolites phosphocholine (*P*Cho), glycerophosphocholine (Gro*P*Cho) and CDP-choline (CDPCho) were confirmed as described previously (Dunlop & Metz, 1989).

Prostaglandin production. Preliminary studies indicated that E-series prostaglandins were major metabolites formed by CHOwt and CHO 11 cells after incubation with exogenous [1-^{14}C]arachidonic acid. Therefore, total immunoreactive prostaglandin (PGE) activity was measured in the incubation medium of cells incubated in the absence of radiolabelled compounds by specific radioimmunoassay. [5,6,8,11,12,14,15 (n)-H]PGE_2 (sp. radioactivity 140–170 Ci/mol) was used as tracer, and an anti-PGE_2 antibody with 100% cross-reactivity for PGE_2 and PGE_1 (Advanced Magnetics rabbit anti-PGE_2 antibody) was used at a final dilution of 1:21000. Antibody-bound prostaglandin was separated from the free ligand with dextran-coated charcoal. The sensitivity of this assay was 7 pg/100 μl (Hill & Larkins, 1989).

Protein content. After removal of incubation medium, cells were solubilized in 0.75 M-NaOH and total protein was determined with Coomassie Brilliant Blue dye (Bradford, 1976).

Statistical analysis. Statistical comparisons were performed by Student's *t* test for unpaired samples, significance being taken at the 5% level ($P < 0.05$) unless indicated otherwise.

RESULTS

EGF-R internalization

Preliminary data (Clark & Hsuan, 1989) had suggested that the EGF-R expressed in CHO 11 cells was defective in EGF-stimulated receptor internalization. To confirm this, we measured the internalization of EGF-R in both CHOwt and CHO 11 cells over 1 h at 37 °C. Bound ^{125}I-EGF was internalized rapidly into CHOwt cells, reaching a maximum of 80% of the specific binding at about 30 min. In contrast, the internalization of bound ^{125}I-EGF into CHO 11 cells was markedly decreased, with only 20% internalized after 30 min (Fig. 1). There was little difference in receptor number (0.5×10^5/cell, CHOwt; 1×10^5/cell, CHO 11) or receptor affinities (2×10^{-10} M and 3×10^{-9} M, CHOwt; 5×10^{-10} M and 4.2×10^{-9} M, CHO 11) between the two cell types.

Phosphorylation of lipocortin I

As it has been suggested that receptor internalization is necessary for phosphorylation of lipocortin I by the EGF-R tyrosine kinase, we examined the response to lipocortin I phosphorylation to EGF in the internalizing (CHOwt) and non-internalizing (CHO 11) cell lines. Our results (not shown) on

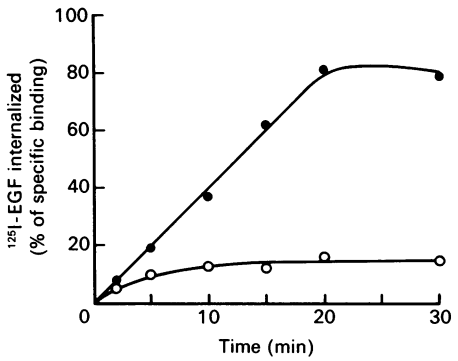

Fig. 1. Internalization of ^{125}I-EGF by CHOwt (●) and CHO 11 (○) cells

Cells were incubated with ^{125}I-EGF (50 ng) for up to 30 min at 37 °C. At various time points, samples were taken and the amount of internalized ^{125}I-EGF was measured by the acid-wash technique as described in the Methods section. The internalized ^{125}I-EGF was calculated as a percentage of the specific binding for each time point. The experiment was repeated twice, and identical results were obtained.

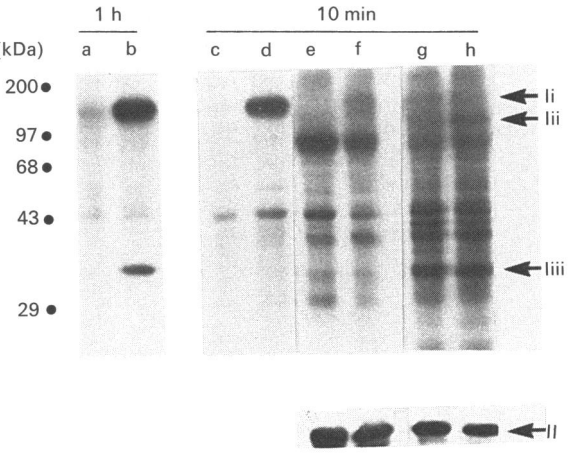

Fig. 2. Phosphorylation of membranes isolated from control or EGF-treated A431, CHOwt and CHO 11 cells

A431 (lanes c, d), CHOwt (lanes e, f) and CHO 11 cells (lanes g, h) were incubated with (lanes d, f, h) or without (lanes c, e, g) 50 ng of EGF/ml for 10 min. In one experiment A431 cells were incubated with (lane a) or without (lane b) 50 ng of EGF/ml for 60 min. Membranes were prepared and phosphorylated as described in the Methods section. Samples were subjected to SDS/PAGE under reducing conditions. The arrows point to the full-length EGF-R (Ii), the truncated EGF-R (Iii) present only in CHO 11 cells and lipcortin I (Iiii). In addition, membrane samples from CHOwt (lanes e, f) and CHO 11 (lanes g, h) cells were immunoblotted with anti-lipocortin I antibody. The autoradiographed band (II) has been cut out and placed under the appropriate lanes. The amount of membrane protein loaded was approx. 20 μg for A431 cells, 25 μg for CHOwt cells and 15 μg for CHO 11 cells.

Table 1. Effect of EGF and exogenous arachidonic acid on PGE production in CHOwt and CHO 11 cells

Values shown are means ± S.E.M. for 3–4 experimental determinations, with the percentage of basal indicated in parentheses: *significant difference from incubation without additions.

Addition	PGE production (pg/10 min per μg of protein)	
	CHOwt	CHO 11
Nil	3.3 ± 0.44	1.78 ± 0.28
EGF (50 ng/ml)	6.5 ± 1.18* (197)	1.56 ± 0.34 (88)
Arachidonic acid (10 μM)	12.30 ± 1.26* (373)	6.42 ± 1.52* (361)

PLA_2 activation in these cells suggest that this effect is rapid and maximal by 10 min. Thus, if phosphorylation of lipocortin I is relevant to PLA_2 activation, it would need to occur within 10 min after EGF stimulation. Cells were treated for 10 min with EGF (50 ng/ml), and membranes were prepared in the presence of Ca^{2+} to maintain the localization of the lipocortin I and phosphorylated as described above. A431 cells were used as a positive control, as the EGF-dependent phosphorylation of lipocortin I has previously been demonstrated in these cells (Fava & Cohen, 1984; Pepinsky & Sinclair, 1986). As the earlier experiments were performed after a 60 min incubation with EGF (Pepinsky & Sinclair, 1986), we repeated these initial conditions (Fig. 2, lanes a, b) in addition to the 10 min incubation (Fig. 2, lances c, d) used for the CHO cell lines. As previously described, stimulation of A431 cells for 60 min with EGF (Fig. 2, lane b) led to the phosphorylation of a band at 35 kDa (Iiii) identified as lipocortin I, in addition to the EGF-R itself (Ii). However, if A431 cells were stimulated for only 10 min with EGF, the autophosphorylation of the EGF-R (lanes c, d: Ii) was still clearly seen, but there was now no evidence of lipocortin I phosphorylation. Similar results were observed for the EGF-R from both CHOwt (lanes e, f) and CHO 11 (lanes g, h) cells. Although the full-length EGF-R (lane f: Ii) and truncated EGF-R (lane h: Iii) were autophosphorylated in response to EGF, there was no EGF-dependent phosphorylation of lipocortin I. It should be noted that there may be constitutive phosphorylation of lipocortin I in CHO 11 cells, a point discussed in more detail below. The level of lipocortin I protein in the CHO cell-lines from samples blotted and probed with an anti-lipocortin I antibody (lanes e–h: II), was comparable in both cell lines. Thus we have been unable to correlate the phosphorylation of lipocortin I with the activation of PLA_2 in CHO or A431 cells.

Prostaglandin production after EGF stimulation

EGF stimulated rapid (within 10 min) formation of PGE in CHOwt cells (197% of basal), but no increase was seen in CHO 11 cells, where PGE production remained at the pre-stimulation level (Table 1). The provision of exogenous arachidonic acid led to a similar increase (3.6-fold) in PGE production in both cell types, indicating that the pathways for PGE synthesis were present in both cell lines.

Distribution of labelled lipid and phospholipids after EGF stimulation

In cells labelled to equilibrium with [1-^{14}C]stearate or [1-^{14}C]arachidonate, it was established that PtdCho was a major labelled species in both CHOwt and CHO 11 cells (23.56 ± 0.84 and 28.0 ± 0.29% of stearate-labelled and 36.9 ± 0.49 and 23.08 ± 0.56% of arachidonic acid-labelled products respectively). EGF stimulation (50 ng/ml) was associated with a rapid (within 10 min) rise (222 ± 24% of basal) in lysophosphatidylcholine (lyso-PtdCho) from [^{14}C]stearate-equilibrium-labelled CHOwt cells (Table 2). Concomitant with this, arachidonate release from [^{14}C]arachidonic acid-labelled CHOwt cells was increased by 165 ± 15%. However, no change in lyso-PtdCho or arachidonic acid release was seen in similarly stimulated CHO 11 cells. Non-transfected CHO cells showed no response to EGF for any of the parameters measured (results not shown).

EGF stimulation of CHOwt or CHO 11 cells had no significant effect on stearate- or arachidonate-labelled phosphoinositides (Table 2) or diacylglycerol production (results not shown). Thus in both CHOwt and CHO 11 cells stimulated with EGF there was no evidence for stimulation of a phosphoinositide-directed PLC. Under identical labelling conditions we were able to elicit an EGF-stimulated increase in diacylglycerol from arachidonic acid-labelled A431 cells (168 ± 12% of basal after 50 ng of EGF/ml, confirming previous observations (Smith *et al.*, 1983).

Phospholipid hydrolysis after melittin addition to CHOwt and CHO 11 cells

The potential for PLA_2 activation could be demonstrated in both CHOwt and CHO 11 cells, where cells pre-labelled with stearic acid and stimulated with a sub-lytic concentration of

Table 2. Effect of EGF (50 μg/ml) or melittin (100 nM) on [^{14}C]stearate- and [^{14}C]arachidonate-labelled lipids in CHOwt and CHO 11 cells

Values are expressed as d.p.m./10 min per mg of protein, as means ± S.E.M. for 4–6 experimental determinations at each condition, with values expressed as percentages of total incorporation indicated in parentheses; *significant difference from value without addition; nd, not detected.

Label	Cells	Addition	Lyso-PtdCho	Fatty acid	Phosphatidylinositols
Stearate	CHOwt	None	926 ± 124 (0.67)	2090 ± 114 (1.5)	22591 ± 294 (15.4)
		EGF	2059 ± 112 (1.50)*	3278 ± 104 (2.4)*	21907 ± 204 (15.9)
		Melittin	4694 ± 304 (3.4)*	3420 ± 174 (2.5)*	13432 ± 314 (9.7)*
	CHO 11	None	977 ± 24 (0.60)	11746 ± 314 (0.72)	16154 ± 311 (9.9)
		EGF	823 ± 33 (0.51)*	15274 ± 414 (9.4)*	16651 ± 294 (10.2)
		Melittin	3676 ± 194 (2.2)*	17214 ± 425 (10.6)*	11476 ± 214 (6.9)*
Arachidonate	CHOwt	None	nd	17857 ± 1032 (18.2)	13345 ± 294 (13.6)
		EGF		29293 ± 2260 (29.6)*	13474 ± 107 (13.7)
		Melittin		36323 ± 1411 (36.8)*	12468 ± 194 (12.6)
	CHO 11	None		59784 ± 5247 (18.7)	50420 ± 214 (15.8)
		EGF	nd	54610 ± 793 (17.2)	51275 ± 179 (16.1)
		Melittin		167944 ± 2141 (52.7)*	40647 ± 341 (12.8)*

Table 3. Effects of EGF, Ca^{2+} ionophore A23187 and OAG on arachidonic acid release from [^{14}C]arachidonic acid-labelled CHOwt and CHO 11 cells

Values are expressed as percentage increase above basal, as mean ± S.E.M. for 3–4 experimental determinations for each condition: *significant difference from incubation without additions; †significant difference from incubation in the absence of EGF. Basal arachidonic acid formation was 17663 ± 1020 and 59805 ± 2142 d.p.m./10 min per mg of protein for CHOwt and CHO 11 cells respectively.

Addition	Cells...	Arachidonic acid release (% of basal) CHOwt	CHO 11
Nil		100	100
EGF (50 ng/ml)		165 ± 13*	91 ± 1
A23187 (2 μM)		204 ± 6*	156 ± 2*
OAG (50 μg/ml)		134 ± 5*	112 ± 3*
EGF + A23187		474 ± 6*†	161 ± 3*
EGF + OAG		405 ± 12*†	117 ± 4*

purified bee-venom melittin (100 nM) increased lyso-PtdCho production significantly (Table 2). Additionally, in both cell types labelled with arachidonic acid, release of labelled arachidonic acid rose on melittin addition. The significant elevation of basal stearate production, apparent in CHO 11 cells compared with CHOwt, was increased further in the presence of melittin, and may indicate some additional hydrolysis at the *sn*-1 position (preferentially labelled by stearate) in CHO 11 cells. After melittin stimulation, phosphoinositides were decreased in both cell types labelled with either stearate or arachidonate (Table 2). As a significant elevation of diacylglycerol in arachidonate-labelled cells was also observed after melittin (from 1079 ± 174 to 1929 ± 171 d.p.m./mg of protein and from 634 ± 24 to 4055 ± 176 d.p.m./mg of protein in CHOwt and CHO 11 cells respectively), this may indicate that an additional activation of PLC contributed a decrease in the phosphoinositides. This was not evident after EGF treatment of either cell type. Taken together, these data support the presence of a PLA_2 mechanism in both CHOwt and CHO 11 cells directed against PtdCho, and possibly against other phospholipids, including phosphoinositides.

Effect of Ca^{2+} ionophore or PKC activation on basal and EGF stimulation of arachidonic acid release in CHOwt and CHO 11 cells

To see if alternative pathways of PLA_2 activation (not involving EGF) were extant in both CHOwt and CHO 11 cells, additional means of stimulation were used. As activation of PLC mechanisms and subsequent Ca^{2+} mobilization, or protein kinase C (PKC) activation, may promote PLA_2 action, the effects of A23187 and OAG on basal and EGF-induced arachidonic acid release were determined in arachidonate-labelled cells. Control incubations contained 0.01% ethanol or 0.01% dimethyl sulphoxide respectively to control for vehicle addition. In both CHOwt and CHO 11 cells, A23187 (2 μM) promoted release of arachidonic acid (Table 3). The effect of EGF was augmented significantly by co-incubation with A23187 in CHOwt cells, but in CHO 11 cells, where no EGF-stimulated arachidonic acid release could be demonstrated, there was no additional effect when A23187 and EGF were combined (Table 3). Similarly, inclusion of the cell-permeant diacylglycerol OAG (50 μg/ml) increased arachidonic acid release from both CHOwt and CHO 11 cells, but significantly augmented the EGF effect only in CHOwt cells.

Hydrolysis of [^{14}C]choline-labelled phospholipid (PtdCho) and formation of water-soluble metabolites in CHOwt and CHO 11 cells

As indicated by stearate-labelling studies, PtdCho formed a major substrate for CHOwt-cell EGF-activated PLA_2, with production of lyso-PtdCho. To investigate this more fully, cells were labelled to steady state with [^{14}C]choline. Additionally, as our results indicate a potential for a constitutive lipocortin I phosphorylation in CHO 11 cells, but not CHOwt cells, evidence was sought for constitutive increases in PLA_2-derived water-soluble metabolites (Gro*P*Cho and Cho) in CHO 11 cells.

The effect of EGF stimulation on choline-labelled CHOwt and CHO 11 cells is presented in Table 4. CHO 11 cells have a significantly increased labelling efficiency when compared with CHOwt cells (2496 ± 214 versus 1420 ± 171 d.p.m./μg of protein). Irrespective of this, lyso-PtdCho production follows EGF addition in CHOwt but not in CHO 11 cells, confirming the activation of a PLA_2 in these cells, as seen previously with fatty acid labelling (Table 2). However, irrespective of EGF stimulation, considerable differences were found between the cell types in water-soluble metabolites after steady-state labelling under basal conditions (Table 4). The percentage of *P*Cho was significantly increased in CHOwt cells, whereas labelled Cho and Gro*P*Cho were significantly increased in CHO 11 cells. However, no evidence was found for EGF-stimulated increases in these water-soluble metabolites to support any combined PLA_2/PLA_1 or PLC action in either cell type.

DISCUSSION

The present study uses two criteria for activation of PLA_2 in EGF-stimulated CHO cells. One establishes hydrolysis of PtdCho to produce lyso-PtdCho, providing direct evidence of a specific action of PLA_2 to remove fatty acid from the *sn*-2-position of PtdCho. The second confirms this by generation of arachidonic acid from equilibrium-labelled cells and measurements of subsequent prostaglandin formation. The findings indicate that PLA_2 activation can be elicited by EGF in CHO cells containing the human EGF holoreceptor (CHOwt), but cannot be demonstrated in CHO 11 cells, transfected with a *C*-terminal truncated form of this receptor (nor in non-transfected CHO cells). In both cell types, however, a Ca^{2+}-dependent activation of PLA_2, as evidenced by arachidonic acid release, could be shown by ionophore stimulation. This is in agreement with data suggesting a Ca^{2+}-dependent PLA_2 activation in other cell types, including platelets (Billah *et al.*, 1980), renal medulla (Craven & De Rubertis, 1983) and mesangial cells (Schlondorff *et al.*, 1985; Boneventre & Swindler, 1988).

A further means of activation was investigated by the addition of OAG. The action of OAG to stimulate arachidonic acid formation in both cell types may be via PKC, as considerable evidence exists for an action of PKC to regulate PLA_2 activity (Ho & Klein, 1987; Parker *et al.*, 1987; Boneventre & Swindler, 1988; Halenda *et al.*, 1989). This may be by a direct action on the enzyme (Halenda *et al.*, 1989) or by an action of OAG to promote translocation of PLA_2 to a membrane site for activation, as shown in cultured macrophages, and further shown to be correlated with translocation of PKC (Schonhardt & Ferber, 1987). However, stimulation of PLA_2, seen as lyso-PtdCho formation in CHOwt cells after EGF stimulation, was not accompanied by a concomitant increase in arachidonic acid-labelled diacylglycerol, which may indicate that PKC stimulation by diacylglycerol is not a pre-requisite for activation under these

Table 4. Distribution of ^{14}C in lipid and water-soluble metabolites of CHOwt and CHO 11 cells preincubated with [^{14}C]choline

Values shown are calculated as percentages of total choline incorporation, as mean ± S.E.M. for 4–6 experimental determinations at each point: *significant difference from CHOwt without added EGF; †significant difference from CHOwt under equivalent conditions; ‡significant difference from CHO 11 without added EGF.

	[^{14}C]Choline incorporation (% of total)			
	CHOwt		CHO 11	
	No EGF	+EGF	No EGF	+EGF
PtdCho	66.33±0.63	65.60±0.42	84.9±0.49†	84.20±0.21
Lyso-PtdCho	0.38±0.02	0.44±0.01*	0.67±0.02†	0.69±0.01
Cho	0.80±0.02	0.80±0.04	1.15±0.05†	1.19±0.01
*P*Cho	25.55±0.05	25.23±0.09	4.13±0.05†	2.76±0.02‡
Gro*P*Cho	3.19±0.17	3.18±0.21	6.83±0.37†	7.46±0.11
CDPCho	1.40±0.07	1.40±0.07	0.94±0.04†	1.21±0.12

conditions. In support of this, recent studies have shown that activation of PLA_2 after receptor stimulation by bradykinin (Kaya *et al.*, 1989) and EGF (Boneventre *et al.*, 1990) are PKC-independent events, and that with EGF a stable modification of the enzyme (PLA_2) occurs. This may be one of the cellular modifications of PLA_2 which is lacking after EGF stimulation of the non-internalized receptor in the CHO 11 cell. However, activation of PLA_2 was seen in both CHOwt and CHO 11 cells (formation of stearate-labelled lyso-PtdCho and arachidonic acid release) after melittin stimulation. In several models this peptide is a known activator of PLA_2 by facilitating endogenous PLA_2-substrate interaction (Shier, 1979; Argiolas & Pisano, 1983). In both cell types the presence of melittin-stimulated PLA_2 activity cannot be dissociated from possible concurrent PLC activation, shown by loss of phosphoinositides. However, in EGF-stimulated CHOwt cells PLA_2 activation occurs without any evidence of prior PLC-activated phosphoinositide hydrolysis, supportive evidence for the independence of these two events, which often occur in parallel in agonist-stimulated cells (Craven & De Rubertis, 1984; Burch *et al.*, 1986; Slivka & Insel, 1987; Crouch & Lapetina, 1988). A23187 and diacylglycerol synergize with EGF to augment arachidonic acid release in CHOwt cells, a synergism which has been reported previously in EGF-responsive cell types (Aoyagi *et al.*, 1985; Margolis *et al.*, 1988*a*), and may indicate that, although independent, parallel activation of PLC and PLA_2 can enhance this cellular response. Under the same conditions, no evidence for augmentation of arachidonic acid release is seen in CHO 11 cells after EGF treatment, further support for a lack of PLA_2 activation.

A potential mechanism for EGF activation of PLA_2 is via removal of the inhibitory activity of lipocortin I, which can occur once lipocortin I is phosphorylated. The EGF receptor may modulate this PLA_2 inhibition, as it has been shown to phosphorylate lipocortin I both *in vivo* and *in vitro* (Pepinsky & Sinclair, 1986). There has been much speculation over whether this plays a physiological role in EGF-dependent PLA_2 activation, but our results would not support this hypothesis. We have shown that the EGF-dependent activation of PLA_2 in CHOwt cells occurs rapidly, whereas no EGF stimulation of lipocortin I phosphorylation is observed under these conditions. Similar findings were obtained after short-term EGF stimulation of A431 cells, even though our own and previous results (Fava & Cohen, 1984; Pepinsky & Sinclair, 1986) demonstrate the EGF-dependent phosphorylation of lipocortin I after 1 h. Thus it seems unlikely that EGF-dependent PLA_2 activation in CHOwt cells is via phosphorylation in lipocortin I. The levels of lipocortin I protein present in the CHOwt membranes suggest that lack of phosphorylation is not related to a decreased level of lipocortin I protein in these cells. It has been postulated that internalization of the EGF-R may be necessary for lipocortin I phosphorylation, although recent evidence based on phosphorylation of lipocortin I by EGF-R in vesicles shed from A431 cells suggests that receptor internalization may not be a prerequisite for this action (Blay *et al.*, 1989). The major difference so far observed between CHOwt and CHO 11 cells is the relative lack of internalization of the truncated EGF-R in CHO 11 cells. Our data would also suggest that receptor internalization is not related to the lack of EGF-stimulated lipocortin I phosphorylation, as no EGF-stimulated lipocortin I phosphorylation is observed in either cell type.

Although comparable with normal cells in many aspects of EGF action, CHO 11 cells do not show EGF-stimulated activation of PLA_2. We propose that this is related to the deletion of a significant functional domain of the receptor, which includes the autophosphorylation sites and the recently identified internalization domain (Chen *et al.*, 1989). The exact deletion point, within the CHO 11 EGF-R, at amino acid 990 is at the end of the region proposed to be required for high-affinity receptor internalization and Ca^{2+} regulation, although we have been unable to demonstrate significant internalization of EGF at either low or high receptor occupancy (S. Clark, unpublished work). We propose two hypotheses for our observed results. The first is that activation of PLA_2 requires internalization of the EGF-R. Although it has been proposed previously that EGF-R internalization is critical for receptor function, it should be noted that most of the effects of EGF can apparently be elicited in its absence. The second is that autophosphorylation of critical sites is required. If activation of PLA_2 required only a functional EGF-R tyrosine kinase, then the EGF-R in both CHO 11 and CHOwt cells should be responsive, but the EGF-R in CHO 11 cells has lost the major sites of receptor autophosphorylation. Although these are thought to play a primary role in kinase activation (Bertics & Gill, 1985; Chen *et al.*, 1989), it is possible they may also be involved in substrate specificity and/or binding, and their loss may therefore interfere with the PLA_2 activation pathway. Further studies are needed to separate the two functions associated with this domain and determine the mechanisms involved. In CHO 11 cells it does not appear that an increase in PGE production is necessary for transduction of a mitogenic signal, as these cells can respond mitogenically to EGF (Clark *et al.*, 1988). The metabolic consequence of failure to elevate PGE in response to EGF remains unclear.

In CHO 11 cells there was some evidence for constitutive phosphorylation of lipocortin I, which could be expected to raise

PLA_2 activity. This was not seen as an increase in basal lyso-PtdCho in these cells, but there was evidence for an increased pathway of PtdCho degradation, which in other cell types proceeds through sequential formation of lyso-PtdCho, Gro*P*Cho and ultimately glycerophosphate and Cho (Morash *et al.*, 1988); basal Gro*P*Cho and Cho were increased in CHO 11 cells. Additionally, the observed decrease in the water-soluble metabolite *P*Cho and an increased labelling efficiency of PtdCho formation in these cells, compared with CHOwt cells, is consistent with an increase in PtdCho-synthetic capacity (George *et al.*, 1989). These effects occur unrelated to EGF stimulation, but in a cell in which constitutive phosphorylation of lipocortin I is demonstrated. A possible explanation is a parallel constitutive increase in PLA_2 followed by rapid removal of fatty acid from lyso-PtdCho formed, leading to increase in Gro*P*Cho. Further experimental support for this is seen in an elevated level of stearic acid in CHO 11 cells, indicating release of stearate from the *sn*-1 position of phospholipids. However, a possible constitutive increase in arachidonic (and stearic) acid is not seen as an increase in PGE. Whether these effects are related to the highly selective clonal nature of these CHO cells, increased hydrolysis of lyso-PtdCho to Gro*P*Cho has been shown in some clonal neuroblastoma lines (Morash *et al.*, 1989), or are related specifically to transfection with the modified human EGF-R, is unknown.

In summary, EGF-stimulated PLA_2 activity can be demonstrated without evidence of prior PLC activation in CHOwt cells. However, cells containing the truncated EGF-R (CHO 11) show no EGF-dependent activation of PLA_2. We propose that this may be related either to their inability to undergo EGF-induced receptor internalization or to the lack of autophosphorylation of critical residues. Furthermore, the experimental findings give no support for the short-term regulation of EGF-stimulated PLA_2 by means of lipocortin I phosphorylation.

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