The tyrosine kinase activity of the epidermal-growth-factor receptor is necessary for phospholipase A₂ activation

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We have previously reported that epidermal growth factor (EGF) activates phospholipase A₂ (PLA₂) independently of phospholipase C (PLC) in renal mesangial cells. In this study we use NIH 3T3 cell lines transfected with the normal EGF receptor (HER14 cells) or with EGF receptor defective in tyrosine kinase activity (K721A cells), to determine whether the intrinsic tyrosine kinase activity of the EGF receptor is required for the PLC-independent activation of PLA₂. Intact cells were preincubated with EGF or other ligands, and then PLA₂ activity was assayed in cell-free extracts with 1-stearoyl-2-[¹⁴C]arachidonyl phosphatidylcholine as the substrate. In HER14 cells, EGF increased PLA₂ activity by 226 ± 30 %, and the tumour promoter phorbol myristate acetate (PMA) increased activity by 223 ± 30 %. The effect of EGF was not mediated through protein kinase C (PKC), whose activation by EGF requires tyrosine kinase activity, since raising intracellular Ca²⁺ alone with the Ca²⁺ ionophore A23187 did not mimic its effect, and the effect of EGF persisted in PKC-down-regulated cells. In K721A cells EGF was ineffective, whereas PMA was still active. Furthermore, in intact HER14 cells prelabelled with [¹⁴C]arachidonate, EGF-stimulated release of [¹⁴C]arachidonic acid was synergistic with A23187, but was unaccompanied by a rise in [¹⁴C]diacylglycerol. EGF had no effect on [¹⁴C]arachidonic acid release in intact K721A cells. We conclude that the tyrosine kinase activity of the EGF receptor is necessary for the PLC-independent stimulation of PLA₂ by EGF.

INTRODUCTION

The metabolites of arachidonic acid are involved in a wide variety of biological processes, including cell growth, regulation of vascular-smooth-muscle tone, inflammation, immune responses, transport and secretion [1]. The release of arachidonic acid from the *sn*-2 position of multiple classes of membrane phospholipids is thought to be the rate-limiting step in eicosanoid production [2,3]. Several mechanisms are postulated to account for ligand-stimulated arachidonic acid release. Some of these involve PLC [4–6], but PLC activation is not necessary for release of arachidonic acid by PLA₂ [7,8]. For example, stimulation of PLC by various ligands results in a rise in intracellular Ca²⁺ which is sufficient to activate PLA₂, and in activation of PKC, which further augments PLA₂ activation in the face of raised intracellular Ca²⁺ [9–13]. However, activation of PLA₂ can occur in a manner that is independent of PKC and not solely explained by a rise in intracellular Ca²⁺ [14,15].

We have previously shown that EGF has a profound synergistic action with Ca²⁺-mobilizing hormones in the release of arachidonic acid and the subsequent elaboration of prostaglandins in renal glomerular mesangial cells in culture [16,17]. These are smooth-muscle-like cells of the glomerulus whose contractile response to vasoactive hormones regulates in part the ultrafiltration process [18]. The effect of EGF required concomitant elevation of intracellular Ca²⁺ for PLA₂ activity, but could be dissociated entirely from PLC activation. Furthermore, studies utilizing dual labelling of membrane phospholipids and the measurement of lysophospholipids confirmed that the effect of EGF was to modulate PLA₂ activity [17]. EGF has been shown to stimulate the release of prostaglandins in other cell types as well [19–21].

Many of the actions of EGF have been shown to involve

receptor-mediated self-phosphorylation, followed by tyrosine phosphorylation of exogenous substrates [22,23]. Most intensively studied have been the proliferative effects of EGF, which have been shown to require the intrinsic tyrosine kinase activity of the EGF receptor [24,25]. More recently, attention has focused on some of the shorter-term actions of EGF and on responses other than mitogenesis [26–28], including the effects in glomerular mesangial cells cited above [16,17] and elsewhere [29]. It was therefore of importance to determine whether the intrinsic tyrosine kinase activity of the EGF receptor is necessary for some of these actions as well. To this end we have utilized NIH 3T3 cells transfected with either the normal human EGF receptor (HER14 cells) or with a mutant version of the receptor devoid of tyrosine kinase activity (K721A cells). Since it has already been shown that EGF-mediated stimulation of PLC activity requires the tyrosine kinase activity of the receptor [30,31], it was necessary to assess separately the PLC-independent activation of arachidonic acid release, reflecting PLA₂ activation. Recently an assay for the measurement of hormone-sensitive PLA₂ by using a cell-free extract has been described [13]. Using this assay, in conjunction with studies of arachidonic acid release from intact cells, we show that the tyrosine kinase activity of the EGF receptor is indeed necessary for the PLC-independent activation of PLA₂ by EGF.

EXPERIMENTAL

Materials

Reagents were obtained from the following sources: EGF from Boehringer Mannheim, Dorval, Quebec, Canada; DMEM and calf serum from GIBCO, Grand Island, NY, U.S.A.; 1-stearoyl-2-[¹⁴C]arachidonyl phosphatidylcholine and [¹⁴C]-

Abbreviations used: PKC, protein kinase C; PLA₂, phospholipase A₂; PLC, phospholipase C; EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco's Modified Eagle Medium; HDMEM, Hepes-buffered DMEM.

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arachidonic acid from Amersham, Oakville, Ont., Canada; and phenylmethanesulphonyl fluoride, leupeptin, pepstatin, A23187, Hepes, PMA and other reagents from Sigma, St. Louis, MO, U.S.A.

Cell culture

HER14 and K721A cells have been previously described [24]. Both are derived from NIH-3T3 cells, originally devoid of EGF receptors, and transfected with cDNA for human EGF receptor so as to express 300000 receptors per cell. In HER14 cells transfection is with a cDNA construct for the human EGF receptor with normal intrinsic tyrosine kinase activity. In K721A cells transfection has been with a mutated cDNA construct for the human EGF receptor such that lysine-721 in the ATP-binding site of the EGF receptor has been replaced with alanine, thus eliminating its intrinsic tyrosine kinase activity [31]. Both cell types were maintained in DMEM with 10% (v/v) calf serum, 50 units of penicillin/ml and 50 µg of streptomycin/ml in 5% CO₂ in air at 37 °C.

PLA₂ activity

PLA₂ was measured essentially as previously described [13,32]. HER14 or K721A cells were grown to confluence in Corning 100 mm-diam. dishes. These cells were then washed twice with DMEM buffered with 30 mM-NaHepes, pH 7.4 (HDMEM), and incubated in the same media in the absence of serum for 2 h at 37 °C. After 2 h, stimulatory agonists were added as indicated in the Results section, and the cells incubated for an additional 20 min at 37 °C. The cells were then washed three times in a homogenization buffer consisting of 50 mM-Hepes, pH 7.5 at 25 °C, 250 mM-sucrose, 1 mM-EDTA and 1 mM-EGTA. The cells were then scraped from each dish in 1 ml of homogenization buffer and disrupted with 25 strokes in a tight-fitting Dounce homogenizer containing homogenization buffer and the following added protease inhibitors: 2 mM-phenylmethanesulphonyl fluoride, 40 µg of leupeptin/ml, 40 µg of pepstatin/ml, and 80 µg of aprotinin/ml. Whole cells and nuclei were removed by spinning the homogenate at 1000 *g* for 5 min, and then cell-free extracts were obtained by centrifugation of pooled homogenates for 1 h at 100000 *g* in a Beckman TLA 100 micro-centrifuge (TL100 rotor), and the supernatants were collected. The excess of Ca²⁺ chelators during preparation of the cell-free extract is required to obtain measurable activity in the supernatant fraction, and is thought to dissociate enzyme from the membrane fraction, which correspondingly loses PLA₂ activity [13]. The protein in each extract was determined by the method of Bradford [33] and adjusted with homogenization buffer to approx. 1 mg/ml (range 0.7–1.3 mg/ml). Substrate was prepared by drying down 1-stearoyl-2-[¹⁴C]arachidonyl phosphatidylcholine under N₂ and resuspending in dimethyl sulphoxide by vigorous vortex-mixing. The assay was initiated by adding 34 µl of the cell-free extract to Microfuge tubes containing 2 µl of substrate in dimethyl sulphoxide (final concn. 15 µM) and 4 µl of CaCl₂ in 30 mM-Hepes, pH 7.5. The final concentration of CaCl₂ was 1 mM in excess of the chelators, and, as expected, there was an absolute requirement for addition of Ca²⁺ during the assay for enzyme activity. After 15 min at 37 °C the reaction was stopped by addition of ice-cold ethanol containing 2% (v/v) acetic acid and 1 mg of arachidonic acid/ml. Then 50 µl of this mixture was spotted on Whatman LK6D t.l.c. plates, which were developed in the organic phase of ethyl acetate/iso-octane/water/acetic acid (55:75:100:8, by vol.). Lipids were detected with I₂ vapour. Arachidonic acid (*R_F* = 0.43) was well resolved from phospholipids (origin). In several experiments diacylglycerol was also identified by using an authentic standard. The relevant areas were scraped into 1 ml of water and 10 ml of Beckman Ready

Protein counting fluid and counted for radioactivity in a Beckman LS 1701 liquid-scintillation counter. Results are expressed as pmol of arachidonic acid released/min per mg of protein at a substrate concentration of 15 µM. Less than 10% of substrate was hydrolysed. Blank activity in the absence of cell-free extract was always less than 5% of total activity, and was subtracted for each experiment. Each assay was performed at least in quadruplicate, and results are shown as means ± S.E.M. Statistical comparisons were by Student's *t* test.

Arachidonic acid and diacylglycerol release in intact cells

These were performed as previously outlined [17]. Briefly, HER14 or K721A cells were plated at a density of 300000 cells per 60 mm dish, and 24 h later [¹⁴C]arachidonic acid (0.5 µCi/dish) was added. After an additional 24 h the cells were washed with 4 × 2 ml of HDMEM containing 5 mg of fatty-acid-free BSA/ml and then incubated in HDMEM with 1 mg of BSA/ml in the absence of serum. Incubation with agonists in HDMEM was as outlined in the Results section. Incubations were stopped by aspirating the media and scraping the cells into 5 ml of iced methanol. Phospholipids were extracted into 7.5 ml of chloroform/0.88% KCl solution (2:1, v/v) by a modified Bligh–Dyer extraction [34]. The upper phase was re-extracted with a solution of 5 ml of chloroform plus 50 µl of conc. HCl. The combined lower phases were dried down under N₂, resuspended in chloroform/methanol (2:1, v/v) and spotted on Merck silica-gel 60 plates. Lipids were separated in heptane/isopropyl ether/acetic acid (60:40:3, by vol.), observed under u.v. light, and spots corresponding to non-esterified fatty acid (free arachidonate) and diacylglycerol were scraped into 1 ml of water and counted for radioactivity in 10 ml of scintillation fluid in a Beckman scintillation counter.

RESULTS

PLA₂ activity was assayed directly in cell-free extracts prepared as described in the Experimental section, with [¹⁴C]arachidonyl phosphatidylcholine as substrate. As previously reported for this assay [13], activity displayed a pH optimum in the alkaline range (pH > 8.5), and was linear with time from zero to 20 min, with protein added in the range 0.5–1.5 mg/ml and with substrate added in the range 10–20 µM (results not shown). Pretreatment of HER14 cells before preparation of the cell-free extract with either EGF or with the PKC activator PMA resulted in a more than doubling of activity (Table 1). In contrast, simply raising intracellular Ca²⁺ by using the Ca²⁺ ionophore A23187 in the intact cells had no effect on PLA₂ activity measured in the cell-free extract, although A23187 is effective in release arachidonic acid from intact cells (see results pertaining to Table 3 below). No increase in label was detected in the diacylglycerol spot.

Table 1. Effect of EGF, PMA and A23187 on PLA₂ activity in HER14 cells

PLA₂ activity was determined as indicated in the Experimental section in cell-free extracts of HER14 cells after stimulation of the cells with EGF (16 nM), PMA (300 ng/ml) or A23187 (1 µM) for 20 min at 37 °C. Values represent means ± S.E.M. for 12 determinations in four separate experiments.

Condition	PLA ₂ activity (pmol/min per mg)
Basal	39.5 ± 1.8
EGF	89.4 ± 11.8 (<i>P</i> < 0.01 versus basal)
PMA	88.2 ± 10.6 (<i>P</i> < 0.01 versus basal)
A23187	44.0 ± 6.0 (Not significant versus basal)

Table 2. Effect of PKC down-regulation on EGF-stimulated PLA₂ activity

HER14 cells were preincubated in the absence (control) or presence (down-regulated) of PMA (300 ng/ml) in 5% serum for 16 h and then in serum-free media for 2 h, and then incubated either in the absence of agonists (basal) or in the presence of EGF (16 nM) or PMA (100 ng/ml). PLA₂ activity was measured in cell-free extracts as outlined in the Experimental section. Values represent means $\pm$ S.E.M. for 12 determinations in three experiments; *P* values refer to comparisons with the corresponding basal (NS, not significant).

Cell	Condition	PLA ₂ activity (pmol/min per mg)
Control	Basal	30.6 $\pm$ 2.1
	EGF	52.0 $\pm$ 4.7 (<i>P</i> < 0.01)
	PMA	52.0 $\pm$ 5.3 (<i>P</i> < 0.01)
Down-regulated	Basal	18.8 $\pm$ 1.6
	EGF	27.3 $\pm$ 2.4 (<i>P</i> < 0.01)
	PMA	18.8 $\pm$ 2.0 (NS)

Table 3. PLA₂ activity in cell-free extracts from mutant K721A cells

PLA₂ activity was determined as described in the Experimental section in cell-free extracts of K721A cells after stimulation of the cells with EGF (16 nM) or PMA (300 ng/ml) for 20 min at 37 °C. Results are expressed as percentages of basal activity, which was 33.4 $\pm$ 3.3 pmol of arachidonate/min per mg of protein (*n* = 12; **P* < 0.01 versus basal)

Stimulatory agonist	Activity (% of basal)
PMA	162 $\pm$ 11*
EGF	100 $\pm$ 5

In order to rule out an effect of EGF mediated through PKC, the effect of EGF was tested in HER14 cells in which PKC had been down-regulated by prolonged prior exposure to PMA as previously described [16,35–37]. Cells were incubated overnight in the absence or presence of 300 ng of PMA/ml in 5% serum. Incubation in decreased serum concentration is required for optimal PKC down-regulation, and results in a slight diminution in PLA₂ activation even in non-down-regulated cells, compared with full (10%) serum. Nevertheless, in down-regulated cells, there was still a significant effect of EGF, although, as expected, the response to the acute addition of PMA was lost (Table 2).

In order to test the dependence of EGF stimulation of PLA₂ activity on the intrinsic tyrosine kinase activity of the receptor, corresponding experiments were conducted in K721A cells. Basal PLA₂ activity in cell-free extracts derived from K721A cells was comparable with that in HER14 cells, and exposure of the cells to PMA resulted in a significant stimulation of this activity (Table 3). In contrast, no stimulation was evident with EGF, suggesting that stimulation of PLA₂ activity by EGF is indeed dependent on the intact tyrosine kinase activity of its receptor.

To determine whether these differences between the HER14 and K721A cells in PLA₂ activity in cell-free extracts did indeed reflect PLA₂ activity responsible for hormone-stimulated release of arachidonic acid in the intact cell, experiments were conducted in cells that had been prelabelled with [¹⁴C]arachidonic acid. As indicated in Table 4, EGF alone doubled release of [¹⁴C]arachidonate-labelled non-esterified fatty acid from HER14 cells, and had a pronounced further synergistic effect with

Table 4. Effect of EGF and A23187 on arachidonate release in HER14 and K721A cells

HER14 or K721A cells were labelled with [¹⁴C]arachidonic acid for 24 h and then incubated for 20 min without added agonist, followed by a further 5 min without (basal) or with (A23187) 1 mM-A23187, or alternatively for 20 min with EGF, followed by a further 5 min without (EGF) or with (EGF + A23187) 1 μ M-A23187. Subsequent steps are as outlined in the Experimental section, and results are expressed as percentages of total phospholipid radioactivity (% c.p.m.) as non-esterified fatty acid (means $\pm$ S.E.M. for six dishes in two experiments): **P* < 0.01 versus basal; †*P* < 0.01 versus A23187.

Cells	Incubation condition	% c.p.m.
HER14	Basal	0.35 $\pm$ 0.04
	A23187	4.72 $\pm$ 0.15*
	EGF	0.81 $\pm$ 0.11*
	EGF + A23187	14.55 $\pm$ 0.18*†
K721A	Basal	0.28 $\pm$ 0.02
	A23187	2.31 $\pm$ 0.18*
	EGF	0.27 $\pm$ 0.02
	EGF + A23187	2.54 $\pm$ 0.21*

A23187. In contrast, in the mutant K721A cells with a defective intrinsic tyrosine kinase, even though A23187 was clearly effective in stimulating release of arachidonic acid, EGF alone caused no increase, nor did EGF augment A23187-induced arachidonic acid release. EGF-induced stimulation of arachidonic acid release was not accompanied by a corresponding increase in release of [¹⁴C]arachidonate-labelled diacylglycerol (results not shown).

DISCUSSION

Many, but not all, of the actions of EGF in a variety of cell types have been shown to depend on intact intrinsic tyrosine kinase activity of the EGF receptor. These include effects on gene transcription and cellular proliferation, enhancement of glucose and amino acid uptake, stimulation of Na⁺/H⁺ exchange and Ca²⁺ influx, and stimulation of PLC activity [24,25,30,31], but not expression and modulation of high- and low-affinity binding sites nor constitutive recycling of the receptor [38]. Another important action of EGF in various cell types is to stimulate the release of arachidonic acid and its metabolites [16,17,19–21]. This effect is of importance in some of the non-mitogenic actions of EGF, particularly in vascular smooth muscle and in the kidney, and arachidonic acid metabolites may also be of importance in mitogenic responses [27,28,39]. Therefore it is important to determine whether this action of EGF is also dependent on the intrinsic tyrosine kinase activity of the receptor.

Site-directed mutagenesis has been used to express in cells, otherwise lacking the EGF receptor, mutant versions of the human EGF receptor devoid of its intrinsic tyrosine kinase activity [24]. As noted above, in cells bearing the mutant receptor, EGF-mediated stimulation of PLC is lost compared with cells expressing abundant normal receptor [30,31]. Such loss of EGF-mediated activation of PLC would be expected to result also in loss of any component of arachidonic acid release which derives from or is dependent on PLC activation. However, a PLC-independent component of arachidonic acid release, attributable to PLA₂ activation, occurs in response to EGF and other agonists in a variety of systems [14–17]. Therefore in the current study we have shown not only that EGF stimulation of arachidonic acid release is lost in tyrosine kinase-deficient mutants, but that this reflects loss also of a PLC-independent component

of arachidonic acid release, most likely representing PLA₂ activation.

In particular, in this study a recently reported [13] assay of PLA₂ activity in cell-free extracts was used. In this assay Ca²⁺ concentration, pH and other conditions are held constant during the assay, and concerns with lipid reacylation and diacylglycerol lipolysis are eliminated. The PLA₂ activity measured in this assay was hormone-sensitive, in agreement with previous reports in other systems [13], as pretreatment of the cells with EGF resulted in a doubling of subsequent activity in the extract. Although the PLA₂ activity in the extract was Ca²⁺-sensitive, as expected, simply raising intracellular Ca²⁺ in the intact cell did not result in an increase in PLA₂ activity in the subsequent cell-free extract, in which the Ca²⁺ concentration was held constant. Activation of PKC in the intact cell by acute exposure to PMA also resulted in a doubling of PLA₂ activity in the cell-free extract. However the effect of EGF did not depend on PKC, as it persisted in cells in which PKC had been down-regulated and in which PMA itself was no longer effective. Therefore, this effect of EGF most likely represents activation of PLA₂ by some covalent modification of the enzyme or of an associated modulatory protein, which persists in the cell-free extract used to assay activity. This stimulation of PLA₂ activity appears also to depend on the intrinsic tyrosine kinase activity of the receptor, as it was lost in K721A cells, even though PMA-mediated stimulation of PLA₂ activity persisted.

It should be noted that coupling of the EGF receptor to PLC and PLA₂ may be a function of receptor number. In cells which over-express the EGF receptor, addition of EGF itself may be sufficient to activate PLC [40,41]. In cells which do not over-express the EGF receptor, and in which PLC activation is not evident, concomitant addition either of a PLC-stimulating and Ca²⁺-mobilizing agonist or of a Ca²⁺ ionophore is required to demonstrate the further stimulation of PLA₂ by EGF [16,17,42]. In the current study, EGF itself was sufficient to cause a small release of arachidonic acid from intact HER14 cells prelabelled with [¹⁴C]arachidonic acid, but in addition EGF very markedly augmented arachidonic acid release in the presence of Ca²⁺ ionophore. Although both basal and Ca²⁺-ionophore-stimulated arachidonic acid release were slightly lower in K721A cells, the effect of EGF under both of these conditions was lost entirely, consistent with the results obtained in cell-free extracts and indicating that a PLC-independent component of EGF-stimulated arachidonic acid release requires the intrinsic tyrosine kinase activity of the receptor.

It should be noted that PLC- and PKC-independent activation of PLA₂ by EGF is in agreement with our previous studies using renal mesangial cells, in which we have shown no effect of EGF on PLC activity, yet a very potent effect of EGF to release arachidonic acid and lysophospholipids [17]. Other results also support the concept that PLA₂ can be activated independently of PLC by hormones that are also capable of stimulating PLC. Using neomycin as an inhibitor of PLC, it was shown in MDCK cells that bradykinin can stimulate the release of arachidonic acid via PLA₂, at least in part independently of PLC [15]. The time course of arachidonic acid release in endothelial cells by bradykinin compared with inositol trisphosphate release also argues that PLA₂ can be activated independently of PLC [14].

The results of this study motivate the search for potential substrates for tyrosine phosphorylation, involved in the modulation of PLA₂ activity. Studies in permeabilized cells and in membrane preparations have provided evidence implicating guanine-nucleotide-binding proteins in PLA₂ activation [43]. In particular, the cellular *ras* oncogene product has been proposed to modulate PLA₂ activity, based on micro-injection studies [44]. Some guanine-nucleotide-binding proteins have been shown to

undergo tyrosine phosphorylation *in vitro* [45], but at present it is not known which guanine-nucleotide-binding proteins interact with PLA₂ in intact cells and whether tyrosine phosphorylation actually influences their biological function *in vivo*. Other substrates for the EGF-receptor tyrosine kinase have been implicated as modulators of PLA₂ activity, but their role in intact cells remains to be defined [45–47]. EGF-stimulated serine and threonine kinases could also conceivably be involved in PLA₂ activation [48]. For PLC, direct phosphorylation by the EGF-receptor kinase has been demonstrated, and has been proposed to account for its activation by EGF [41,49]. An analogous effect to phosphorylate PLA₂ directly is particularly attractive, in view of the observation that EGF stimulation of PLA₂ persists or is 'remembered' in cell-free extracts derived from EGF-stimulated cells. However, antibodies are not yet available which would allow more definitive evaluation of this possibility. The findings in the current study motivate such investigation, and appropriate further studies using the model system outlined in this study should serve to clarify these questions.

H.J.G. is a fellow and M.M.V. a student of the Kidney Foundation of Canada, and B.L.M. is a fellow of the Medical Research Council of Canada. This work was supported by grants from the Kidney Foundation of Canada, the Heart and Stroke Foundation of Ontario, and the Physicians of Ontario to K.L.S.

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Received 9 October 1987/27 November 1989; accepted 4 January 1990