Characterization and identification of an epidermal-growth-factor-activated phospholipase A₂

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The production of arachidonic acid (AA), which is involved in mitogenic signalling by epidermal growth factor (EGF), is most directly accomplished by the action of phospholipase A₂ (PLA₂). We demonstrate that EGF treatment of intact NEF cells rapidly activates a cytosolic PLA₂, as measured in cell-free extracts by the release of radiolabelled AA from exogenously added 1-stearoyl-2-[1-¹⁴C]arachidonoyl phosphatidylcholine. Activation of PLA₂ by EGF resulted in an enhanced V_{max} , and no change in K_m . The PLA₂ activity was eluted in a single peak at 0.4 M-NaCl from a Mono Q anion-exchange column, and migrated with an approximate molecular mass of 70 kDa on a Superose 12 gel-filtration column. The EGF-activated PLA₂ activity co-migrated with the basal PLA₂ activity upon gel filtration, and persisted after partial purification, which indicates that the activation is due to a stable modification of the enzyme. The EGF-stimulated PLA₂ is Ca²⁺-dependent, with maximal activity at micromolar concentrations of Ca²⁺, has a pH optimum at 9, associates with the particulate cell fraction in a Ca²⁺-dependent fashion, and is selective for arachidonoyl at the *sn*-2 position. These data demonstrate the EGF-induced activation of a PLA₂, which is similar to a recently cloned high-molecular-mass AA-selective cytosolic PLA₂, thus providing a link between EGF-receptor tyrosine kinase activation and AA metabolism.

INTRODUCTION

Epidermal growth factor (EGF) is a polypeptide that modulates proliferation and differentiation of a wide variety of cell types, by binding to its receptor (EGF-R) [1]. The EGF-R is a transmembrane glycoprotein of 170 kDa with protein tyrosine kinase activity [2]. EGF binding induces dimerization of the EGF-R, and consequently the EGF-R tyrosine kinase is activated [3–5], causing phosphorylation of the receptor itself as well as several substrates [5,6]. Using EGF-R mutants, the protein tyrosine kinase activity was shown to be essential for all receptor-mediated responses involved in mitogenic signalling [7–10]. Several EGF-R substrates and/or associated proteins have been identified, including *ras* GTPase-activating protein (GAP), phosphatidylinositol 3-kinase, and phospholipase C γ (PLC) [5,11]. The tyrosine phosphorylation of PLC γ enhances its catalytic activity [12], which results in hydrolysis of phosphatidylinositol 4,5-bisphosphate, thus generating inositol 1,4,5-trisphosphate and diacylglycerol (DG). These important intracellular second messengers mediate the release of Ca²⁺ from intracellular stores, and activate protein kinase C (PKC), respectively [13,14]. Furthermore, EGF stimulates the release of arachidonic acid (AA) and eicosanoids in several cell types [15–20]. Interestingly, the formation of AA metabolites has been shown to be required for EGF-induced mitogenic signalling [17,19].

AA and its metabolites (e.g. prostaglandins, leukotrienes and thromboxanes) play an important role in a wide variety of biological processes [21–23]. The production of AA can be accomplished by the sequential activation of PLC and DG lipase [24,25]. Most directly, however, the release of AA is regulated by phospholipase A₂ (PLA₂) [21,22]. PLA₂ hydrolyses membrane phospholipids, including those containing an arachidonoyl group

at the *sn*-2 position, thus producing lysophospholipids and AA. PLA₂s are ubiquitous in nature and can be detected in almost all cell types [21]. Several different types of PLA₂ have been identified and purified; extracellular pancreas and venom types, as well as various cellular types, which all have a typical low molecular mass of 14–18 kDa ([26,27], and references therein). Recently, however, the purification and identification has been reported of a class of high-molecular-mass (60–110 kDa) cytosolic PLA₂s (cPLA₂) from rat mesangial cells [28], rat kidney [29], human monocytic U937 cells [30–32], mouse macrophage RAW264.7 cells [33] and rabbit and bovine platelets [34,35]. In addition to their higher molecular mass, these cPLA₂s can be distinguished from the low-molecular-mass PLA₂s on the basis of several characteristics: they have arachidonoyl-group selectivity, are active at physiological Ca²⁺ concentrations, and show Ca²⁺-dependent translocation from the cytosol to the cell membrane. The cDNA encoding the cPLA₂ of human monocytic U937 cells has been cloned and sequenced recently [36, 37], which elicited a molecular mass of 85.2 kDa and showed high homology with cPLA₂ of mouse macrophage RAW264.7 cells. Interestingly, the sequence analysis demonstrated the presence of a Ca²⁺-dependent lipid-binding domain with homology to PKC, GAP and PLC [36], as well as several potential kinase phosphorylation sites [37]. Therefore the cPLA₂s are likely candidates for playing a role in signal transduction.

Recently, it has been shown that EGF enhances PLA₂ activity in glomerular mesangial cells [38] and NIH 3T3 fibroblasts transfected with the EGF-R [39,40]. The EGF-induced PLA₂ activation requires EGF-R tyrosine kinase activity, but not EGF-R autophosphorylation [39,40]. However, the identity as well as the biochemical characteristics of the EGF-activated PLA₂ remain to be established. In this study we report the rapid activation of PLA₂ upon EGF treatment of different cell lines.

Abbreviations used: EGF, epidermal growth factor; EGF-R, EGF receptor; PLC, phospholipase C; DG, diacylglycerol; PKC, protein kinase C; AA, arachidonic acid; PLA₂, phospholipase A₂; cPLA₂, cytosolic PLA₂; FCS, fetal-calf serum; PMSF, phenylmethanesulphonyl fluoride.

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Upon partial purification, this EGF-stimulated PLA₂ was identified as a high-molecular-mass (70 kDa) PLA₂, with biochemical and chromatographic characteristics resembling those of previously purified and cloned cPLA₂s. Furthermore, the EGF-induced PLA₂ activation persists after partial purification of the enzyme, indicating that the activation is due to a direct stable modification that may involve the phosphorylation of this PLA₂.

EXPERIMENTAL

Materials

EGF (receptor grade) was obtained from Biomedical Technologies Inc. (Stoughton, MA, U.S.A.); 1-stearoyl-2-[1-¹⁴C]-arachidonoyl phosphatidylcholine (54 mCi/mmol) and 1-palmitoyl-2-[1-¹⁴C]oleoyl phosphatidylcholine (52.2 mCi/mmol) were from Amersham International (Amersham, Bucks., U.K.); the Mono Q HR 5/5 anion-exchange column, the Superose 12 HR 10/30 gel-filtration column, and the molecular-mass markers were from Pharmacia LKB Biotechnology (Uppsala, Sweden); and the silica gel, Silic AR (60 Å), was from Mallinckrodt (St. Louis, MO, U.S.A.). The substrate 1-acyl-2-[1-¹⁴C]linoleoyl phosphatidylethanolamine was prepared as described previously [41].

Cell culture

NEF and HER 14 cells, kindly provided by Dr. J. Schlessinger (Department of Pharmacology, New York University Medical Centre, New York, NY, U.S.A.), are mouse NIH 3T3 fibroblasts devoid of endogenous EGF-R, transfected with the human EGF-R cDNA. The NEF cells express approx. 300 000 receptors [42], and HER 14 cells express approx. 200 000 [8,9]. The NEF, HER 14, Rat-1 (rat fibroblasts) and A431 (human epidermoid carcinoma) cells were routinely grown in Dulbecco's modified Eagle's medium supplemented with 7.5% (v/v) fetal-calf serum (FCS), buffered with 40 mM-NaHCO₃ under a 7.5%-CO₂ atmosphere.

Preparation of cell extracts for PLA₂ activity assays

Sub-confluent cultures of the different cell types, grown in either six-well tissue-culture clusters or 75 cm² or 165 cm² flasks (Costar) and kept for 16 h in the absence of FCS before stimulation, were stimulated with 200 ng of EGF/ml. After the appropriate incubation period, the medium was discarded and cells were washed twice with cold Ca²⁺- and Mg²⁺-free phosphate-buffered saline. Subsequently, the cells were scraped at 4 °C in 1 ml (75 or 165 cm² flask) or 400 µl (six-wells plate) of homogenization buffer, containing 50 mM-Tris/HCl (pH 7.5), 50 mM-NaF, 200 µM-Na₃VO₄, 1 mM-phenylmethanesulphonyl fluoride (PMSF), 100 units of aprotinin/ml, 5 mM-benzamidine, and 100 µg of trypsin inhibitor/ml, and further disrupted with 20 strokes of a Potter-Elvehjem motor-driven pestle. For preparation of supernatant fractions the homogenate was centrifuged at 15 000 g for 20 min.

PLA₂ activity assay

PLA₂ activity was measured *in vitro* by the release of radio-labelled arachidonic acid from the *sn*-2 position of 1-stearoyl-2-[1-¹⁴C]arachidonoyl phosphatidylcholine. The radiolabelled phosphatidylcholine was dried under N₂ and then dispersed in water by freezing and thawing, followed by sonication for 3 × 20 s under N₂. The assay-incubation mixture (final volume 200 µl) contained 0.2 M-Tris (pH 9), 1 mM-CaCl₂, 5 µM radiolabelled phosphatidylcholine (110 000 d.p.m.) and approx. 25 µg of protein from the cell-free extract, and was incubated for 3 min at 37 °C in a shaking water bath. Under these assay conditions,

always less than 5% of the substrate was hydrolysed. The reaction was stopped by addition of 2.5 ml of Dole mixture (propan-2-ol/n-heptane/H₂SO₄, 40:10:1, by vol.). Upon mixing, 1.5 ml of water and 1.5 ml of n-heptane were added and the solution was mixed again. Of the heptane phase 1 ml was applied to a silica-gel column (200 mg) that was washed with 1 ml of diethyl ether to elute the remaining non-esterified fatty acids. Upon addition of scintillation fluid, the released radiolabelled AA was measured by liquid-scintillation spectrometry. To correct for background hydrolysis, the reaction was performed in the absence of protein. The amount of protein in the samples was determined with a BCA protein assay (Pierce).

Partial purification of PLA₂

For Mono Q anion-exchange chromatography, cell-extract supernatant fractions (obtained from cells cultured in two 165 cm² flasks) were prepared after scraping the cells in 4 ml of homogenization buffer, containing 20 mM-Tris (pH 7.4), 1 mM-EDTA, 10% glycerol, 50 mM-NaF, 200 µM-Na₃VO₄, 1 mM-PMSF, 5 mM-benzamidine and 100 µg of trypsin inhibitor/ml at 4 °C. The sample (4 ml) was applied to a Mono Q anion-exchange column, equilibrated and run with 50 µM-Hepes (pH 7.5)/1 mM-EDTA/1 mM-EGTA/10% glycerol. The column was run at a flow rate of 0.5 ml/min and the fraction size was 1 ml. When no further protein could be detected by the A₂₈₀, the bound proteins were eluted with a 20 ml linear gradient of 0–1 M-NaCl. Of each fraction 100 µl was assayed for PLA₂ activity in the presence of 2 mM-CaCl₂ (1 mM-CaCl₂ in excess of chelators). The PLA₂ activity was eluted in a single peak at approx. 0.4 M-NaCl. The recovery from this column was 70%, with an approx. 12-fold purification of the PLA₂ activity in the peak fraction compared with the cell-extract supernatant. A sample (500 µl) of the peak fraction obtained after the Mono Q anion-exchange column was applied to a Superose 12 gel-filtration column.

For direct Superose 12 gel filtration, cell-extract supernatant fractions (cells were grown in 165 cm² flasks) were prepared after scraping the cells in 1 ml of homogenization buffer, containing 20 mM-Tris (pH 7.4), 1 mM-EDTA, 1 M-KCl, 10% glycerol, 50 mM-NaF, 200 µM-Na₃VO₄, 1 mM-PMSF, 100 units of aprotinin/ml, 5 mM-benzamidine and 100 µg of trypsin inhibitor/ml at 4 °C. A sample of the supernatant fraction (500 µl) was loaded on to the Superose 12 gel-filtration column, which was equilibrated and run in the buffer mentioned above, with a flow rate of 0.5 ml/min [for Superose 12 gel filtration of the Mono Q peak fraction, the column was equilibrated and run in 20 mM-Tris/HCl (pH 7.4)/1 mM-EDTA/10% glycerol/1 M-KCl]. Fractions (0.5 ml) were collected in plastic tubes containing 5 µl of BSA (100 mg/ml), and 100 µl of each fraction was assayed for PLA₂ activity, as described above. For the Ca²⁺- and pH-dependency experiments, 50 µl of the peak fraction was assayed at each Ca²⁺ and pH value. The recovery of the enzyme activity was approx. 100% with a 10-fold purification in the peak fraction compared with the cell-extract supernatant. The molecular mass of the enzyme was estimated by using IgG (150 kDa), BSA (67 kDa) and cytochrome *c* (12.5 kDa) as molecular-mass markers (Pharmacia LKB Biotechnology). Essentially the same results were obtained with a buffer containing 0.5 M- instead of 1 M-KCl.

RESULTS

Kinetics of EGF-induced PLA₂ activation

PLA₂ activity was measured in an assay *in vitro* by the release of AA from exogenously added phosphatidylcholine, radio-labelled at the *sn*-2 position with [1-¹⁴C]arachidonoyl, as de-

Fig. 1. Kinetics of basal and EGF-stimulated PLA₂

Cell-free extract of NEF cells, either treated with 200 ng of EGF/ml for 10 min (●) or untreated (○), were assayed for PLA₂ activity as described in the Experimental section, except that either incubation time (a) or substrate concentration (b) was varied. In (a) PLA₂ activity was assayed as a function of time. The reaction mixture contained 5 μM substrate in an initial volume of 2.5 ml. After the indicated incubation periods (0.5–40 min), 200 μl samples were removed and mixed with 2.5 ml of Dole mix in order to stop the reaction. In (b) PLA₂ activity was assayed as a function of substrate concentration. The reaction was performed for 3 min in the presence of the indicated substrate concentrations (0.25–5 μM). The inset is a Lineweaver–Burk plot of the data. The results from a representative experiment are shown.

scribed in the Experimental section. Analysis of the kinetics of PLA₂ activity in extracts of EGF-stimulated and unstimulated NEF cells as a function of time revealed non-linear reaction kinetics, especially at longer assay periods (Fig. 1a), although only maximally 5% of the substrate was converted. Similar results have been described for the cPLA₂ activity in mouse macrophage RAW264.7 cells [43]. However, the reaction was linear with time for about 5 min, and the initial velocity of the reaction was higher for PLA₂ obtained from EGF-stimulated cells than for unstimulated cells (Fig. 1a). Therefore all PLA₂ activity assays are performed at 3 min incubation periods.

Subsequently, PLA₂ activity was assayed for 3 min as a function of substrate concentration in accordance with Michaelis–Menten (Fig. 1b). Plotting the data in accordance with Eisenthal & Cornish-Bowden [43a] revealed that the EGF-stimulated PLA₂ showed the same K_m value (2.5 μM) and

Fig. 2. Mono Q anion-exchange chromatography profile of the PLA₂ activity

NEF cells were extracted as described in the Experimental section and the extracts were applied to a Mono Q column. If no more protein could be washed off the column (fractions 10–20), as detected by the A_{280} , a 0–1 M-NaCl linear gradient was run to elute bound proteins (fractions 20–40). The collected fractions were assayed for PLA₂ activity as described in the Experimental section. Similar results were obtained in several experiments.

an average 2-fold increased V_{max} (200 pmol/min per mg) as compared with basal PLA₂ activity, and subsequent Lineweaver–Burk analysis of the data revealed linear reaction kinetics (Fig. 1b, inset). This indicates that only a single type of PLA₂ is detected in our assays. In these experiments, NEF cells were stimulated with EGF for 10 min, since PLA₂ activation was maximal after 10 min of EGF treatment, resulting in an average 2.2-fold stimulation (2.17 ± 0.16 , $n = 19$), and decreased after longer incubation with EGF (results not shown). Furthermore, enhanced PLA₂ activity was also detected after a 10 min EGF treatment of Rat-1, HER 14 and A431 cells (results not shown).

Partial purification and identification of the PLA₂ activity

To characterize and identify the PLA₂ activity in the NEF cells, the supernatant of cell extracts was fractionated by sequential Mono Q anion-exchange chromatography and Superose 12 gel filtration. The recovered fractions were assayed for 30 min in order to obtain optimal detection of the PLA₂ activity present in these fractions. As the extract was applied to the Mono Q column (Fig. 2), no enzymic activity was detected in the flow-through of the column, but a single peak of activity was eluted at 0.4 M-NaCl. The peak fraction of the recovered activity from the Mono Q column was further characterized by Superose 12 gel filtration. All enzymic activity migrated as a single peak at a molecular mass of approx. 70 kDa (Fig. 3). In the peak fraction obtained after Superose 12, the PLA₂ activity was purified approx. 250-fold compared with the cell homogenate, and the recovery from the sequentially used columns was 67% and, on average, 100% respectively. These data strongly indicate that the detected PLA₂ belongs to the class of high-molecular-mass cPLA₂s, since these have been reported to bind to a Mono Q anion-exchange column and to migrate with a molecular mass of 60–70 kDa on a Superose 12 gel-filtration column [28–38], in contrast with the low-molecular-mass PLA₂s. Furthermore, by using radiolabelled phosphatidylethanolamine, which is a more suitable substrate for the low-molecular-mass PLA₂s, and 10 mM-Ca²⁺ in the assay (optimal for most low-molecular-mass PLA₂s) [27], no indications of the presence of these PLA₂s were found in the fractions obtained after anion-exchange or gel-filtration column chromatography. In addition, immunoblotting of the peak fractions with a polyclonal anti-(low-molecular-mass cellular PLA₂) antibody [44] demonstrated the absence of this type of PLA₂ (result not shown).

Fig. 3. Superose 12 gel-filtration profile of the PLA₂ activity purified by Mono Q column chromatography

The recovered peak fraction of the Mono Q-purified PLA₂ (fraction 28) was partially loaded on to a Superose 12 gel-filtration column. After gel filtration the fractions were collected and assayed for PLA₂ activity as described in the Experimental section. The arrows indicate the recovery of the molecular-mass markers of 150 kDa (IgG), 67 kDa (BSA) and 12.5 kDa (cytochrome *c*). Similar results were obtained in several experiments.

Fig. 4. Superose 12 gel-filtration profile of basal and EGF-activated PLA₂ activity

NEF cells were incubated in the absence (○) or presence (●) of 200 ng of EGF/ml for 10 min, and extracted in a 1 M-KCl buffer as described in the Experimental section. After extraction, the extract supernatants were applied to a Superose 12 gel-filtration column and the recovered fractions were assayed for PLA₂ activity. The arrows indicate the positions of recovery of the molecular-mass markers of 150 kDa (IgG), 67 kDa (BSA) and 12.5 kDa (cytochrome *c*). Similar results were obtained in ten different experiments.

Fig. 5. Substrate-selectivity of the PLA₂

NEF-cell extracts were assayed for PLA₂ activity in a volume of 1 ml with either 1-stearoyl-2-[1-¹⁴C]arachidonoyl phosphatidylcholine (●) or 1-palmitoyl-2-[1-¹⁴C]oleoyl phosphatidylcholine (○). At the indicated times, 200 μ l samples were removed and the release of radiolabelled fatty acid was measured as described in the Experimental section. The data represent mean values of a duplicate experiment.

Table 1. Ca²⁺-dependent membrane association of PLA₂

NEF cells were extracted in the presence of 1 mM-EDTA and 1 mM-EGTA (EDTA/EGTA) or 1 mM-CaCl₂ (Ca²⁺) as indicated. The cell homogenates were centrifuged and the PLA₂ activity in the supernatant (S) or pellet (P) fractions was determined in the presence of 1 mM-CaCl₂ in excess of chelators, as described in the Experimental section. The data, from a representative experiment, are presented as the percentage of the PLA₂ activity of the total extracts, recovered in each fraction.

Addition	PLA ₂ activity (% of total)	
	P	S
EDTA/EGTA	8	92
Ca ²⁺	84	16

Identification of the EGF-activated PLA₂

To investigate whether the EGF-activated PLA₂ in the NEF cells is identical with the PLA₂ detected in Figs. 2 and 3, high-salt-extract supernatants of EGF-treated and untreated NEF cells were applied directly to the Superose 12 gel-filtration column. The recovered fractions were assayed for 3 min to detect optimal differences between fractions obtained from EGF-stimulated and unstimulated cells. As shown in Fig. 4, basal as well as EGF-stimulated PLA₂ activity migrated in the same single peak at a molecular mass of approx. 70 kDa. No additional activity was detected in the gel-filtration fractions obtained from the extract of the EGF-stimulated cells. A 2-fold higher peak of PLA₂ activity was recovered from extracts of EGF-treated cells compared with control cells, demonstrating that the EGF-induced PLA₂ activation persists after partial purification of the enzyme. The detection of both unstimulated as well as EGF-stimulated PLA₂ in the same molecular-mass fraction indicates that the regulation of the PLA₂ activity is not due to the association or dissociation of a regulatory protein. Lineweaver-Burk analysis of both PLA₂ peak fractions revealed linear reaction kinetics and gave essentially the same results as obtained with cell-free extracts: no difference in *K_m* values between both fractions and a 2-fold EGF-enhanced *V_{max}*. (results not shown). Taken together, the EGF-activated PLA₂ is identical with the PLA₂ activity as presented in Figs. 2 and 3, and is the only type of PLA₂ detected in the activity assays.

Characterization of the EGF-activated PLA₂

To identify and characterize the EGF-activated PLA₂ further, we investigated some of the biochemical properties known to be characteristic of the high-molecular-mass cytosolic PLA₂s, thereby discriminating them from the low-molecular-mass PLA₂s.

sn-2 acyl-chain-selectivity of the PLA₂ was determined by examination of the time course of hydrolysis of PC containing either [1-¹⁴C]arachidonoyl or [1-¹⁴C]oleoyl at the *sn*-2 position. The results in Fig. 5 indicate that the PLA₂ preferentially hydrolysed phospholipids with AA esterified at the *sn*-2 position. Furthermore, for both substrates the PLA₂ displayed non-linear reaction kinetics.

When the cells were extracted in the absence of Ca²⁺ (i.e. in the presence of 1 mM-EDTA and 1 mM-EGTA), the main PLA₂ activity was found in the supernatant fraction after centrifugation (Table 1). However, extraction of the cells in the presence of 1 mM-Ca²⁺ resulted in the recovery of enzymic activity mainly in the particulate fraction. It is noteworthy that, already in the absence of exogenously added Ca²⁺ and without the presence of

Fig. 6. Effect of Ca²⁺ and pH on PLA₂ activity

NEF-cell extract supernatant was applied to Superose 12 gel filtration. The recovered fractions were assayed for PLA₂ activity as described in the Experimental section. Subsequently, the fraction containing highest activity was assayed under standard assay conditions except for the Ca²⁺ concentration (a) and the pH (b) during the assay. In (a), the Ca²⁺-dependency was determined by performing the PLA₂ activity assay either in the presence of 1 mM-EDTA and 1 mM-EGTA or in the presence of different concentrations of CaCl₂ at pH 9, as indicated. In (b), the effect of pH was determined by performing the PLA₂ assay at different pH values, based on the pH of the added Tris/HCl buffer at 1 mM-CaCl₂. In both panels the results from a representative experiment are shown.

EDTA/EGTA, most of the PLA₂ activity is lost from the supernatant fraction (results not shown). These observations suggest that the enzyme is translocated from the cytosol to the membrane by means of enhanced cellular Ca²⁺ concentrations, as has been reported for the high-molecular-mass cPLA₂s in previous studies [31,33,36,45,46]. Increasing the ionic strength of the extraction buffer by adding 1 M-KCl resulted in a further enhancement of the recovery of PLA₂ activity in the supernatant fraction (results not shown).

The PLA₂ activity from the NEF cells, partially purified by gel filtration, is absolutely dependent on the presence of Ca²⁺. As shown in Fig. 6(a), in the presence of 1 mM-EGTA and 1 mM-EDTA no enzymic activity could be detected. Maximal PLA₂ activity was already observed at micromolar concentrations of Ca²⁺. The enzymic activity showed no significant increase in the presence of free Ca²⁺ over the range 10 μM–1 mM, whereas above 1 mM the PLA₂ activity was decreased. Since other studies have not found such a decrease in PLA₂ activity at high Ca²⁺ concentrations, the decrease of phospholipid hydrolysis may very well be due to an effect on structural organization of the substrate. In addition, the pH-dependency of the partially purified NEF cell PLA₂ is shown in Fig. 6(b). The highest PLA₂ activity was observed in the alkaline range, with an optimum at pH 9; however, the activity could also be detected at physiological pH values. The observed enhancement of the PLA₂ activity as a consequence of the increase in Ca²⁺ and pH at physiological values may provide a regulatory mechanism for cellular PLA₂ activity, since EGF treatment of most cells results in an enhancement of intracellular Ca²⁺ and pH [5,10,11].

Taken together, these data demonstrate that, on the basis of its chromatographic and biochemical characteristics, the EGF-activated PLA₂ in the NEF cells is similar to several recently reported high-molecular-mass cPLA₂s.

DISCUSSION

Identification and characterization of signal-transduction pathways involved in mitogenic signalling is of great importance in understanding the regulation of proliferation of normal and transformed cells. Recent studies have indicated that AA and its metabolites are second messengers in EGF-induced signal transduction [17,19]. AA release is accomplished most directly by means of PLA₂. Therefore, we investigated the identity and biochemical characteristics of the EGF-activated PLA₂. We demonstrate that: (i) EGF rapidly activates a cytosolic PLA₂ by increasing its *V*_{max}; (ii) the EGF-activated PLA₂ has a molecular mass of approx. 70 kDa, as determined by gel filtration; (iii) the EGF-activated PLA₂ has biochemical and chromatographic characteristics similar to those of recently purified and cloned cPLA₂s; and (iv) EGF-stimulated PLA₂ activity persists in cell extracts and partially purified preparations, indicating that the EGF-induced activation of the PLA₂ is due to a direct stable modification of the enzyme.

In previous studies, PLA₂ activation by different stimuli was often determined by measuring the release of radiolabelled AA or its metabolites (e.g. prostaglandins) from intact cells. This approach, however, is indirect and therefore less suitable, since the sequential action of PLC and DG lipase or other indirect effect cannot be excluded (e.g. stimulation or inhibition of AA- and eicosanoid-metabolizing enzymes, inhibition of substrate competitors, or heterogeneous labelling of the phospholipid pool). In the present study, the activation of PLA₂ upon EGF treatment of cells was established by means of an assay *in vitro* in which the hydrolysis of exogenously added 1-stearoyl-2-[1-¹⁴C]arachidonoyl phosphatidylcholine was measured in cell-free extracts. Furthermore, the observation of enhanced enzymic activity after partial purification of the PLA₂ from EGF-stimulated cells unequivocally demonstrated EGF-induced activation of PLA₂. Kinetic analysis of the PLA₂ activity revealed an enhanced *V*_{max}, but no change in *K*_m upon EGF treatment of the cells.

From the results obtained after Superose 12 gel filtration, we estimated the molecular mass of the EGF-activated PLA₂ to be approx. 70 kDa. However, it cannot be excluded that the higher molecular mass of this PLA₂ is due to a tightly associated protein, although the cell extracts were made at a 1 M-KCl concentration, which strongly decreases this possibility. Final determination of the molecular mass would require further purification and SDS/PAGE of the enzyme. Nevertheless, in addition to its high molecular mass, the EGF-activated PLA₂ in our study and the recently described cPLA₂s are very similar with regard to pH optimum, Ca²⁺-dependency and maximal activity at micromolar Ca²⁺ concentrations, Ca²⁺-dependent membrane association, arachidonoyl-group selectivity and elution from Mono Q at 0.4 M-NaCl [28–38], all this in contrast with the low-molecular-mass PLA₂s. These cPLA₂s all have a molecular mass of 60–70 kDa as determined by gel filtration; however, upon SDS/PAGE in most cases the cPLA₂ turned out to be a 100–110 kDa protein, whereas cloning revealed a molecular mass of 85.2 kDa [36]. The observations that these cPLA₂s are AA-selective, respond to physiological increases in intracellular Ca²⁺ and pH with enhanced activity, are translocated to the membrane in a Ca²⁺-dependent manner, and that the cDNA for one of these cPLA₂s revealed a Ca²⁺-dependent lipid-binding domain as well as several potential kinase phosphorylation sites (for both

serine/threonine and tyrosine kinases), renders these high-molecular-mass cPLA₂s to be likely candidates for playing an important role in signal transduction [36,37].

Several events have been implicated in activation of cellular PLA₂, including the activation of G-proteins or PKC [38,39,47–53], or the enhancement of intracellular pH or Ca²⁺ [31,33,36,45,46]. Indeed, the observed enhancement of the PLA₂ activity as a consequence of the increases in Ca²⁺ and pH at physiological values may provide a regulatory mechanism for cellular PLA₂ activity, since EGF treatment of most cells results in an enhancement of intracellular Ca²⁺ and pH [5,10,11]. However, none of these activation mechanisms seems to account for the (total) EGF-induced PLA₂ activation [18,38,39], although the involvement of a G-protein has been reported in some studies [18,54]. Our study shows that the EGF-induced PLA₂ activation is detectable in cell extracts, and can still be measured after partial purification. Furthermore, EGF-activated PLA₂ was recovered from a gel-filtration column without a concomitant shift in molecular mass. This implies that, at least in these activated fractions, Ca²⁺, pH, or the association or dissociation of a regulatory protein (e.g. G-protein subunits), are not directly involved in the observed EGF-induced activation of PLA₂. In fact, these data indicate that a direct stable modification of the enzyme results in its activation. Furthermore, the PLA₂ activity revealed an enhanced V_{max} , but no change in K_m upon EGF treatment of the cells, which is in agreement with an EGF-induced stable modification of PLA₂ at a site other than the catalytic site. In addition, the enhancement of PLA₂ activity by EGF requires EGF-R tyrosine kinase activity [39], and the cloned cPLA₂ contains several potential kinase phosphorylation sites [37]. Therefore, these data legitimize the suggestion that EGF-induced phosphorylation of cPLA₂, most likely on tyrosine residues, may enhance its catalytic activity.

As a consequence of the EGF-induced activation of this AA-selective cPLA₂, enhanced release of AA will occur, thus enabling the formation of higher levels of AA metabolites too. Interestingly, data from our laboratory demonstrate that activation of PLA₂ and the subsequent formation of the AA metabolite leukotriene C₄ is essential in the EGF-induced Ca²⁺ influx and *jun* B expression [55]. This opens the intriguing possibility that EGF-R-induced phosphorylation activates the cPLA₂, causing AA release and the subsequent leukotriene C₄ production, due to which enhanced Ca²⁺ influx occurs resulting in enhanced PLA₂ translocation to the cell membrane. Furthermore, the AA metabolism was shown to be involved in EGF-induced *c-myc* and *c-fos* expression in BALB/c 3T3 fibroblasts and rat mesangial cells respectively [17,19]. More generally, AA and/or the eicosanoids have been reported to activate PKC [56], activate different ion channels [57], and cause the release of Ca²⁺ from intracellular stores [58]. Furthermore, they inhibit the GTPase-activating protein [59,60] and stimulate a GTPase-inhibiting protein [61], both regulators of *ras* p21 GTPase activity. Since evidence has been provided that the *ras* protein is implicated in growth-factor receptor (e.g. EGF-R) tyrosine kinase-mediated mitogenic signalling [62–64], PLA₂ activation may provide an important link between the growth-factor- and the *ras* signal-transduction pathways.

In conclusion, our results demonstrate the EGF-induced activation of a high-molecular-mass, AA-selective, cytosolic PLA₂. The data indicate that a direct stable modification of this cPLA₂, possibly tyrosine phosphorylation, enhances its enzymic activity. The subsequently released AA and AA metabolites may exert effects on a variety of events involved in regulation of cell proliferation and differentiation.

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