Distinct structural specificities for functional coupling of the epidermal growth factor receptor to calcium-signalling versus phospholipase A₂ responses

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Activation of phospholipase C (PLC), leading to a rise in cytosolic Ca²⁺, and of phospholipase A₂ (PLA₂), leading to a release of arachidonic acid, are among the early transmembrane signalling events that have been demonstrated in response to occupancy of the epidermal growth factor (EGF) receptor. The tyrosine kinase activity of the receptor has been shown to be necessary for both of these responses. This requirement for the tyrosine kinase activity could conceivably implicate a role for receptor autophosphorylation in the activation of PLA₂. We now demonstrate that coupling of the EGF receptor to PLA₂ was not impaired in a deletion mutant (CD126) devoid of the 126 amino acids from the C-terminus which include four major autophosphorylation sites. Functional coupling of the EGF receptor to PLA₂ was demonstrated using three different experimental designs: (1) release of [¹⁴C]arachidonic acid from prelabelled intact cells, (2) release of [³H]arachidonic acid from prelabelled cells permeabilized with glass beads, and (3) direct measurement of PLA₂ enzymic activity in cell-free extracts using an 'in vitro' assay employing exogenous phospholipid substrate. Functional coupling of the EGF receptor to PLA₂ occurred despite the absence of a demonstrable Ca²⁺-signalling response and the detection of diminished but persistent PLC-γ phosphorylation on tyrosine residues in the CD126 deletion mutants. These results point to a clear distinction in the biochemical mechanism and role for receptor autophosphorylation in functional coupling of the EGF receptor to PLA₂ activation versus Ca²⁺ signalling.

INTRODUCTION

Activation of receptor tyrosine kinases by their respective ligands leads to enhanced kinase activity resulting in both receptor autophosphorylation and substrate phosphorylation [1]. In the case of the epidermal growth factor (EGF) receptor, autophosphorylation appears to modulate substrate phosphorylation and subsequent cellular responses to EGF [1], but is not essential for the mitogenic response. Activation of phospholipase C (PLC), resulting in a rise in cytosolic Ca²⁺ [2], and of phospholipase A₂ (PLA₂), resulting in release of arachidonic acid [3,4], are among the early transmembrane signalling events that have been demonstrated in response to occupancy of the EGF receptor. PLC-γ serves as a substrate for the EGF receptor tyrosine kinase, and this phosphorylation is postulated to result in activation of PLC [5,6]. In terms of PLA₂, we have demonstrated activation by EGF and consequent release of arachidonic acid independently of PLC [3,4], and have shown that PLA₂ activation also requires the intrinsic tyrosine kinase activity of the EGF receptor [7]. This requirement for tyrosine kinase activity could conceivably implicate a role for receptor autophosphorylation in the release of arachidonic acid by PLA₂.

Whereas greater attention has been focused on the role of the PLC response to EGF, this response is confined predominantly to cell types vastly overexpressing the EGF receptor [8]. In contrast, stimulation of arachidonic acid release has been demonstrated in many cell types expressing levels of EGF receptors ambiently found in normal tissues [3,4]. Moreover, it was shown recently that the metabolism of arachidonic acid was necessary

for the mitogenic responses of EGF in BALB/c 3T3 cells [9]. The metabolites of arachidonic acid are involved in a wide variety of biological processes, including mitogenesis, regulation of vascular smooth muscle tone, inflammation, immune responses, transport and secretion [10]. Several mechanisms are postulated to account for ligand-stimulated arachidonic acid release. Some of these involve PLC [11–13], but PLC activation is not necessary for the release of arachidonic acid by PLA₂ [14–16]. We have previously shown that EGF has a profound synergistic action with Ca²⁺-mobilizing hormones in the release of arachidonic acid and in the subsequent generation of prostaglandins in various cell types [3,4,7,16]. Furthermore, by utilizing dual labelling of membrane phospholipids and the measurement of lysophospholipids, we demonstrated that the effect of EGF was to modulate PLA₂ enzymic activity, and excluded a major contributory role for other pathways such as diacylglycerol lipolysis, PLA₁ followed by lysophospholipase, or alterations in lysophospholipid reacylation. This effect of EGF requires a concomitant elevation of intracellular Ca²⁺ for PLA₂ activity, and this rise in cytosolic Ca²⁺ can be brought about by the separate action of a Ca²⁺-mobilizing hormone or Ca²⁺ ionophore.

In the current study, we use a C-terminal deletion mutant of the EGF receptor truncated at amino acid 1060 and missing four of the identified major autophosphorylation sites to delineate the role of receptor autophosphorylation in PLA₂ activation in comparison with its relationship to Ca²⁺ signalling. NIH 3T3 cells devoid of endogenous EGF receptors were transfected with constructs containing cDNAs designed to express either the normal human EGF receptor (HER cells) or a truncated version

Abbreviations used: PLA₂, phospholipase A₂; PLC, phospholipase C; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco's modified Eagle medium.

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of the EGF receptor missing the C-terminal 126 amino acids (CD126 cells). These cells expressed similar numbers of receptors.

EXPERIMENTAL

Materials

Reagents were obtained from the following sources: EGF from Boehringer-Mannheim, Dorval, Quebec, Canada; platelet-derived growth factor (PDGF) from R & D, Minneapolis, MN, U.S.A.; Dulbecco's modified Eagle's medium (DMEM) and calf serum from Gibco, Grand Island, NY, U.S.A.; 1-stearoyl-2-[¹⁴C]arachidonoyl phosphatidylcholine, [¹⁴C]- and [³H]-arachidonic acid from Amersham, Oakville, Ontario, Canada. All other reagents, including phenylmethanesulphonyl fluoride, leupeptin, pepstatin, A23187, Hepes, phorbol 12-myristate 13-acetate (PMA), and glass beads, were from Sigma, St. Louis, MO, U.S.A.

Cell culture

NIH 3T3 cells devoid of endogenous EGF receptors were transfected to express 300000 of the various mutant receptors per cell. In HER cells transfection was with a cDNA construct for the normal human EGF receptor with full intrinsic tyrosine kinase activity [17,18]. In K721A cells transfection was with a mutated construct of the human EGF receptor, such that lysine-721 in the ATP-binding fold was replaced with alanine, thus eliminating its tyrosine kinase activity [17,18]. In CD126 cells cDNA constructs were prepared that deleted 126 amino acids from the C-terminus. These cells expressed normal intrinsic tyrosine kinase activity. All cell types were maintained in DMEM supplemented with 10% calf serum, 50 units of penicillin/ml and 50 µg of streptomycin/ml in 5% CO₂ in air at 37 °C.

Arachidonic acid release in intact cells

For arachidonic acid release using intact cells, cells were plated at a density of 300000 cells per 60 mm dish, and 24 h later were labelled with [¹⁴C]arachidonic acid. After an additional 24 h the cells were washed 4 times with DMEM buffered with 30 mM-Hepes (pH 7.4) containing 5 mg of fatty-acid free BSA/ml, and then were incubated in DMEM/30 mM-Hepes with 1 mg of BSA/ml in the absence of serum. Incubations were stopped by aspirating the medium and scraping into 5 ml of ice-cold methanol. Phospholipids were extracted into 7.5 ml of chloroform/0.88% KCl (2:1, v/v) as previously reported [3]. The upper phase was re-extracted with a solution of 5 ml of chloroform and 50 µl of concentrated HCl. The combined lower phase was concentrated and spotted on to silica gel 60 plates (Merck). Lipids were separated in heptane/di-isopropyl ether/glacial acetic acid (60:40:3, by vol.), visualized under u.v. light and spots corresponding to non-esterified fatty acid (free arachidonate) and diacylglycerol were scraped into 1.0 ml of water and counted for radioactivity in 10 ml of scintillation fluid in a Beckman scintillation counter.

Arachidonic acid release in glass-bead-permeabilized cells

This was conducted as previously described by Hack *et al.* [16]. Briefly, cells were plated at a density of 300000 cells per 3.5 cm-dish. At 48 h the medium was replaced with medium containing [³H]arachidonic acid (1 µCi/dish). Experiments were conducted after 72 h of culture, at which time the cells were confluent. The cells were washed with 4 × 2 ml of 30 mM-Hepes-buffered DMEM, pH 7.4, containing 2 mg of fatty-acid-free BSA/ml at 37 °C. Incubations were carried out in the same medium containing 1 mg of defatted albumin/ml at 37 °C. For

permeabilization, glass beads (300 mg/dish), prepared as described by Hack *et al.* [16], were added and the dish was placed on a Specimix (Sybron Inc.) and allowed two complete rocks. The dishes were then carefully removed from the Specimix and allowed to stand for 5 min. Control incubations were carried out in the same way but without the addition of glass beads. For measurement of [³H]arachidonate release into the medium, at the end of incubations the medium was removed with a syringe and filtered through a 0.2 µm-pore-size Acrodisc-13 filter, and samples were added to liquid scintillation fluid for counting of radioactivity. In preliminary experiments the effect of varying the external Ca²⁺ concentration on arachidonic acid release was determined by using various Ca²⁺ and EGTA concentrations in the permeabilization solution [19]. There was no release of arachidonic acid at calculated external Ca²⁺ concentrations below 100 nM, with a maximal effect occurring at a calculated external Ca²⁺ concentration in the permeabilization solution of 1 µM, corresponding to Ca²⁺ concentrations that are achieved in intact cells in response to Ca²⁺-mobilizing hormones or Ca²⁺ ionophore.

PLA₂ activity

Recently, an assay for the measurement of hormone-sensitive phospholipase A₂ in a cell-free extract has been reported [20]. For this assay, cells were pretreated with the agonist of interest and a cytosolic fraction was prepared in the presence of excess Ca²⁺ chelators which are required to transfer measurable activity from the membrane fraction, which correspondingly loses PLA₂ activity to the cytosolic fraction. Ca²⁺ is then re-added to the separated cytosol fraction as there is an absolute Ca²⁺ requirement for PLA₂ enzymic activity. The activity is measured as the release of [¹⁴C]arachidonic acid from exogenously added labelled substrate as previously described [7].

Immunoprecipitation and immunostaining of PLC-γ

Cells grown to confluence in 10 cm culture dishes were stimulated with EGF for 3 min at 37 °C and scraped directly into lysis buffer containing 50 mM-Hepes (pH 7.5), 150 mM-NaCl, 10% (w/v) glycerol, 1% Triton X-100, 1.5 mM-MgCl₂, 1 mM-EGTA, 200 µM-sodium orthovanadate and 30 mM-*p*-nitrophenyl phosphate. Immunoprecipitation was performed utilizing a rabbit polyclonal anti-PLC-γ antibody coupled to Protein A-Sepharose (Sigma) as previously described by Margolis *et al.* [6]. Immunoprecipitates were separated on a 10% (w/v) polyacrylamide gel, they were then transferred to nitrocellulose and immunostaining was performed with the anti-phosphotyrosine antibody PY20 (ICN) [21]. Blots were incubated with goat anti-rabbit antibody linked to alkaline phosphatase and developed as reported by Hack *et al.* [16].

Fluorescent dye loading and Ca²⁺ determination

Confluent monolayers of cells were suspended by brief exposure to trypsin (0.5 g/l) and EDTA (0.2 g/l) in phosphate-buffered saline, followed by the addition of trypsin inhibitor and cell recovery in a nutrient medium as previously described [22]. Indo-1-acetoxymethyl ester (2.5 µM) was added for 30 min at 37 °C, after which the cells were washed free of extracellular dye. Fluorescence measurements were performed in a Hepes-buffered physiological saline solution at an excitation wavelength of 355 nm, with emission recorded at 400 nm. Calibration and determination of cytosolic Ca²⁺ concentrations were carried out as previously reported [22,23].

Data presentation and statistics

Where indicated, statistical comparisons were made using Student's *t* test for paired and unpaired data. Differences were

considered significant when $P < 0.05$. Data are presented as means $\pm$ S.E.M.

RESULTS AND DISCUSSION

In the first set of studies, we examined the effect of EGF on arachidonic acid release from intact [¹⁴C]arachidonate-labelled CD126 cells. Cells were labelled to equilibrium, incubated without or with Ca²⁺ ionophore (A23187) or EGF, and [¹⁴C]-labelled non-esterified fatty acid, representing free arachidonate, was separated by t.l.c. as previously described [3]. Table 1 shows that EGF alone caused a small release of arachidonic acid but very markedly augmented the response to A23187. EGF did not alter the intracellular Ca²⁺ response to the Ca²⁺ ionophore (results not shown; [3]). This pattern of response is similar to that which we have previously reported using the same stimulatory conditions in HER cells [7]. These results suggest that the C-terminus of the EGF receptor is not required for functional coupling of the receptor to phospholipase A₂. Further corroboration was obtained using two other experimental approaches, namely cell permeabilization and measurement of PLA₂ enzymic activity in cell-free extracts. In the cell permeabilization studies, in addition to using HER and CD126 cells, studies were also carried out in the parent NIH 3T3 cells devoid of EGF receptors, as well as in DK721 cells expressing an EGF receptor mutant which is deficient in tyrosine kinase activity by virtue of the substitution of alanine for lysine at position 721 in the ATP-binding fold of the receptor, as previously reported by Honegger *et al.* [17,18]. Cells were prelabelled with [³H]arachidonic acid for 48 h and then permeabilized and incubated in the absence or presence of EGF, and release of [³H]arachidonic acid was measured as outlined in the Experimental section. As shown in Table 2, EGF was completely ineffective in eliciting a release of arachidonic acid in NIH 3T3 and DK721A cells, whereas in both HER and CD126 cells EGF caused a near doubling of arachidonic acid release.

The results of PLA₂ measurements in cell-free extracts are shown in Table 3. Pretreatment of CD126 cells with EGF and preparation of cell-free extracts followed by assay of PLA₂ enzymic activity was carried out as described by Goldberg *et al.*

[7], and demonstrated a significant stimulation of PLA₂ activity in EGF-pretreated CD126 cells compared with non-EGF-pretreated controls. This response in CD126 cells is comparable with that which we have previously reported in HER cells, and is absent in tyrosine-kinase-deficient DK721A cells [7].

It was therefore evident, using three experimental approaches, that deletion of the C-terminus of the EGF receptor, which contains four of the autophosphorylation sites, does not abrogate functional coupling of the EGF receptor to PLA₂ activation. We next examined the relationship to PLC phosphorylation and Ca²⁺ responses in these cells. Physical association of the EGF receptor with direct phosphorylation of PLC- γ has been demonstrated, and this phosphorylation has been postulated but not proven to result in PLC activation and Ca²⁺ signalling [5,6]. In the current study, CD126 cells and HER cells displayed EGF-dependent tyrosine phosphorylation of PLC- γ (Fig. 1). Correspondingly, we examined the Ca²⁺ response to EGF in these cell lines using the Ca²⁺-sensitive fluorescent probe indo-1 (Fig. 2). EGF caused a marked rise in intracellular Ca²⁺ in HER cells. Surprisingly, in CD126 cells, no Ca²⁺-signalling response to EGF was evident. In contrast, PDGF was able to elicit a rise in intracellular Ca²⁺ in these cells, indicating that there was no disruption of the Ca²⁺-mobilizing process in the CD126 cells.

Taken together, these results demonstrate different structural specificities for the functional coupling of the EGF receptor to PLA₂ on the one hand and to the Ca²⁺-signalling response on the other. For both of these responses, the intrinsic tyrosine kinase activity of the EGF receptor has been shown to be necessary [7,24]. However, the role of the C-terminal end of the EGF receptor containing four autophosphorylation sites is quite different for these two responses. In the case of Ca²⁺ signalling, loss of these autophosphorylation sites is associated with phosphorylation of PLC- γ and disappearance of measurable changes in cytosolic Ca²⁺. It should be noted that previous studies [5,6,25] have demonstrated that EGF-dependent physical association of PLC- γ with the EGF receptor accompanies its tyrosine phosphorylation. In these studies, the preferred state of EGF-dependent association of the EGF receptor was with the non-tyrosine phosphorylated form of PLC- γ , as determined by co-immunoprecipitation using antibodies to both proteins. Co-immunoprecipitation also required the intrinsic tyrosine kinase activity of the EGF receptor [24]. Of note in the current study is that Ca²⁺-signalling responses to EGF did not occur in the C-terminal deletion mutant, despite persistent EGF-dependent phosphorylation of PLC- γ (Fig. 1). The EGF-induced Ca²⁺-signalling responses in HER cells were indeed a reflection of release of Ca²⁺ from intracellular stores, in as much as the response persisted in the absence of extracellular Ca²⁺ (results not shown). Furthermore, in HER cells EGF induced a rise in inositol phosphates of $181.6 \pm 26.8\%$ of baseline, in agreement with our previous reports [26], whereas in the CD126 cells EGF did not produce a rise in inositol phosphates over baseline levels (N. Hack & K. L. Skorecki, unpublished work). Nevertheless, it cannot be excluded that other factors besides inositol-phosphate-mediated Ca²⁺ release from intracellular stores contribute to the overall Ca²⁺-signalling response to activation of PLC- γ by receptor tyrosine kinases. This is particularly relevant in view of the recent reports [27,28] demonstrating a lack of correlation between Ins(1,4,5)P₃ generation and a rise in intracellular Ca²⁺.

Previous studies have not focused on the coupling of the EGF receptor to PLA₂ and generally have considered EGF-stimulated arachidonic acid release as part of the PLC response to EGF. However, the results of the current study clearly dissociate these two signalling responses. In contrast to the marked effect of C-terminal deletion on the functional coupling of the EGF receptor to Ca²⁺-signalling, the PLA₂ response remained intact. Of

Table 1. Effects of EGF and Ca²⁺ ionophore A23187 on arachidonic acid release from intact cells

CD126 cells were labelled with [¹⁴C]arachidonic acid for 24 h and then preincubated in the absence (basal, A23187) or presence (EGF, EGF + A23187) of EGF (100 nM) for 20 min, and this was then followed by a further 5 min incubation in the absence (basal, EGF) or presence (A23187, EGF + A23187) of A23187 (1 μ M) for a further 5 min. Non-esterified fatty acids released into the medium and in cells were separated from total phospholipids using t.l.c. as outlined in the Experimental section, and results are expressed as percentages of total phospholipid [¹⁴C] radioactivity (d.p.m.) in non-esterified fatty acid (means $\pm$ S.E.M. for 12 dishes in four separate experiments).

* $P < 0.001$ versus basal; † $P < 0.001$ versus A23187 alone.

Incubation conditions	Arachidonic acid release (%)
Basal	0.24 $\pm$ 0.03
A23187	2.05 $\pm$ 0.29
EGF	1.03 $\pm$ 0.10*
EGF + A23187	9.44 $\pm$ 0.42†

Table 2. Effect of EGF on arachidonic acid release in permeabilized cells

Cells were prelabelled with [^3H]arachidonic acid for 48 h, preincubated in the absence or presence of 100 nM-EGF and 30 mM-Hepes-buffered DMEM containing 1.94 mM- CaCl_2 and 2.0 mM-EGTA (final external free Ca^{2+} concentration 5.0 μM), and then permeabilized with glass beads as described in the Experimental section. Following a further 5 min incubation, 500 μl portions of the medium were removed and ^3H radioactivity was determined. Results for EGF stimulation are expressed as percentages of the corresponding controls (EGF vehicle), and are presented as means $\pm$ S.E.M. for five separate experiments performed in duplicate. * $P < 0.001$ versus NIH 3T3 or K721 cells.

Cell type	Arachidonic acid release (% of control)
NIH 3T3	89.4 $\pm$ 2.7
DK721	93.7 $\pm$ 3.3
HER	168.4 $\pm$ 5.1*
CD126	155.0 $\pm$ 2.5*

Table 3. Effect of EGF on PLA_2 activity in cell-free extracts of CD126 cells

PLA_2 activity (pmol of arachidonate/min per mg of protein) was determined in cell-free extracts derived from CD126 cells as described in the Experimental section. Values represent means $\pm$ S.E.M. of 24 determinations under each stimulatory condition conducted in six separate experiments. * $P < 0.001$ versus basal.

Cell incubation condition	PLA_2 activity
Basal	92.0 $\pm$ 3.7
EGF (100 nM)	189.1 $\pm$ 11.6*

Fig. 1. EGF-dependent tyrosine phosphorylation of PLC- γ in HER and CD126 cells

Cells were grown in 10 cm dishes and incubated for 3 min in the absence or presence of EGF (100 nM). After incubation, lysates were subjected to immunoprecipitation using anti-PLC- γ antibodies as described in the Experimental section. After SDS/PAGE, the proteins were transferred to nitrocellulose and immunostained with the anti-phosphotyrosine antibody PY20. Lanes 1 and 2, CD126 cells; lanes 3 and 4, HER cells.

particular interest is the observation that EGF stimulation of PLA_2 persists in cell-free extracts derived from EGF-stimulated HER and CD126 cells, suggesting a covalent modification of PLA_2 or tight association with a modulatory protein. However, the mechanism for this response is clearly very different from

Fig. 2. Cytosolic Ca^{2+} response of HER and CD126 cells to EGF and PDGF

Fluorescent dye loading, fluorescence measurements and calibration of cytosolic Ca^{2+} were carried out as outlined in the Experimental section. In (a) representative tracings of the responses to EGF and PDGF obtained in HER cells are shown. Summary values for 12 determinations from six experiments were as follows: basal Ca^{2+} , 294.8 $\pm$ 5.6 nM; peak Ca^{2+} , 959.6 $\pm$ 44.1 nM ($P < 0.001$ compared with basal) in response to 100 nM-EGF. In (b) representative tracings of the responses to EGF and PDGF in CD126 cells are shown. Summary values for 12 determinations from six experiments were as follows. The basal cytosolic Ca^{2+} was 253.5 $\pm$ 2.2 nM; this did not rise in response to 100 nM-EGF (220.5 $\pm$ 1.7 nM- Ca^{2+}) but rose to a peak value of 413.3 $\pm$ 6.6 nM in response to 0.3 nM-PDGF ($P < 0.001$ compared with basal or EGF).

that involved in generation of a Ca^{2+} signal, since it does not require the C-terminal end of the EGF receptor containing four autophosphorylation sites.

The failure to separately assess PLA_2 activation in the past may have been due to the fact that a rise in intracellular Ca^{2+} is necessary for PLA_2 activity. However, such a rise in intracellular Ca^{2+} does not necessarily emanate from EGF-mediated phosphorylation and activation of PLC, but rather can result from the concomitant action of other Ca^{2+} -mobilizing agonists such as vasopressin and other neuropeptides [29,30]. This is of particular relevance in the many non-transformed cell types which do not overexpress the EGF receptor, and in which functional coupling of EGF to Ca^{2+} mobilization is not evident [3]. Examples include cells of the glomerular microcirculation, vascular smooth muscle cells and hepatocytes [31–33]. This may reflect the need for high levels of EGF receptors for coupling to Ca^{2+} signalling, whereas for PLA_2 this may not be necessary, perhaps due to different coupling mechanisms. Indeed, evidence obtained in two different cell systems have implicated the potential involvement of a guanine-nucleotide-binding protein (G-protein) in EGF-mediated stimulation of PLA_2 [16,34]. Such an intermediate role for a G-protein in transducing the signal from the occupied EGF receptor to PLA_2 may explain the occurrence of this response as opposed to the PLC response in cells which do not overexpress the EGF receptor. In such cells bearing normal levels of EGF receptor the presence of EGF may prime the cell and markedly augment the arachidonic acid release that occurs in response to the Ca^{2+} -mobilizing agonist. Such a mechanism could provide the arachidonic acid that has been shown to be a requirement for mitogenesis [9]. The results of the current study provide a biochemical basis for understanding the distinction between coupling of the EGF receptor to Ca^{2+} signalling and to PLA_2 .

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