Epidermal growth factor induces rapid and transient association of phospholipase C-γ1 with EGF-receptor and filamentous actin at membrane ruffles of A431 cells

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SUMMARY

Addition of epidermal growth factor to A431 cells results in dramatic changes in cell morphology. Initially the cells form membrane ruffles accompanied by increased actin polymerization, followed by cell rounding. Activation of the tyrosine kinase of the receptor by binding epidermal growth factor leads also to phosphorylation and activation of phospholipase C-γ1, a key enzyme in the phosphoinositide pathway. In this study we have investigated the localization of phospholipase C-γ1 during cell activation by epidermal growth factor. It is shown that addition of the growth factor to A431 cells leads to a translocation of phospholipase C-γ1 from the cytosol to the membrane fraction. Interestingly, this relocation is exclusively directed to the membrane ruffles. Most of the phospholipase C-γ1 associates to the membrane and a small fraction to the underlying skeleton. Immunocytochemical studies demonstrated that phospholipase C-γ1 co-localizes with the epidermal growth factor receptor and also filamentous actin at the membrane ruffles. Moreover, using anti-phosphotyrosine antibodies we found that the membrane ruffles are significantly enriched in phosphotyrosyl proteins. Between 5 and 10 minutes after stimulation the membrane ruffles disappear and also the co-localization of phospholipase C-γ1 with the epidermal growth factor receptor and filamentous actin. These results support the notion that activation of A431 cells by epidermal growth factor leads to the formation of a signalling complex of its receptor, phospholipase C-γ1 and filamentous actin which is primarily localized at membrane ruffles.

Key words: signal transduction, EGF-receptor, phospholipase C-γ1, membrane ruffles, signalling complex

INTRODUCTION

Epidermal growth factor (EGF) is one of the most intensively studied growth factors to date, and many details are known of the molecular features of the factor itself, of its receptor and various components involved in EGF-induced signal transduction (for review see: Yarden and Ulrich, 1988; Cadena and Gill, 1992; Fantl et al., 1993, and references therein). Recently, the cytoskeleton has been indicated to play an intermediate role in EGF induced signal transduction, not only because EGF causes drastic changes in the structure of the cytoskeleton (Schlessinger and Geiger, 1981; Rijken et al., 1991), but also because a variety of components involved in the signal transduction cascade are associated with the cytoskeleton. For example the EGF-receptor has been demonstrated to be associated with the actin microfilament system (van Bergen en Henegouwen et al., 1992). In addition, the activity of a number of key enzymes involved in signal transduction such as phosphatidylinositol kinase, phosphatidyl-4-phosphate kinase, diacylglycerol kinase and phospholipase C (PLC) was demonstrated to be associated with cytoskeletal elements (Payrastre et al., 1991; Grondin et al., 1991; Vaziri and Downes, 1992; Yang et al., 1994).

The most compelling evidence in favour of a role of the cytoskeleton in EGF-induced signal transduction has been obtained by the finding that the EGF-receptor itself is an actin-binding protein (Payrastre et al., 1991; Grondin et al., 1991; Vaziri and Downes, 1992; Yang et al., 1994). The cytoskeleton associated receptors are able to phosphorylate their substrates more efficiently than receptors not associated with the cytoskeleton (Gronowski and Bertics, 1993).

In order to obtain more insight into the possible role of the
cytoskeleton in EGF induced signal transduction, we have studied the localization of one of the key components in the EGF activated signal transduction cascade, i.e. phospholipase C-γ1 (PLC-γ1) during cell activation. PLC-γ1 is a substrate of the EGF-receptor tyrosine kinase and upon phosphorylation its activity is increased (Margolis et al., 1989; Meisenhelder et al., 1989; Nishibe et al., 1989, 1990). A typical feature of the PLC-γ family is the presence of two src homology regions 2 (SH2) and one src homology region 3 (SH3). The SH2 domain has been demonstrated to bind specifically to different phosphotyrosine-containing binding sites located in different regions of growth factor receptors, including the EGF-receptor (Mohammadi et al., 1991; Rotin et al., 1992). SH3 domains have been shown to bind to proline-rich sequences (Yu et al., 1994) and have been identified in several cytoskeletal proteins (Drubin et al., 1990), suggesting that they are involved in regulating the interaction of signal molecules with the cytoskeleton. Using a microinjection approach it has been demonstrated recently that the SH3 domain of PLC-γ is responsible for the association of PLC-γ to the actin microfilament system (Bar-Sagi et al., 1993). This latter would be in agreement with earlier observations that PLC activity is associated with the actin microfilament system (Payrastre et al., 1991). Altogether, these observations suggest that the actin microfilament system comprises a matrix necessary for EGF-induced signal transduction.

The microfilament system undergoes major changes upon EGF stimulation. The stress fibres disappear whereas actin polymerization at the cortex is induced (Rijk et al., 1991). Induction of ruffling of the apical membrane as a consequence of stimulation with growth factors has been described (Chinkers et al., 1979; Boonstra et al., 1987; Mellström et al., 1988; Campos-Gonzalez et al., 1990). Membrane ruffles represent changes in cell morphology and cytoskeletal architecture. The molecular events responsible for ruffle formation are not known, but it is believed that actin polymerization plays a crucial role (Mellström et al., 1988). Evidence has been obtained that the GTP binding protein Rac is required for growth factor induced membrane ruffling (Ridley et al., 1992).

In this paper we demonstrate that EGF causes a redistribution of PLC-γ1 from the cytoplasm to the plasma membrane, targeted to the newly formed membrane ruffles. In these ruffles PLC-γ1 strongly co-localizes with the EGF-receptor and with filamentous actin (F-actin). The membrane ruffles are also the site where the first tyrosine phosphorylated proteins were found after EGF administration. Thereafter the phosphotyrosine signal moved towards the cell-cell interface. Our results lead to the hypothesis that the membrane ruffles are the place where the intracellular signal pathway is initiated.

MATERIAL AND METHODS

Cell culture

A431 (human epidermoid carcinoma) cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal calf serum (Flow Labs, Irvine, Scotland, UK) in a 7% CO2 atmosphere. Cells were grown to about 80% confluency in culture bottles for biochemical experiments and on glass coverslips for immunofluorescence microscopy. The A431 cells were incubated in serum-free medium for 2 to 48 hours (unstimulated) at 37°C, thereafter they were stimulated with 100 ng/ml EGF for the time indicated (EGF stimulated) at 37°C or 4°C.

Assay of phospholipase C activity

Stimulated (10 minutes with 200 ng/ml EGF at 37°C) and unstimulated A431 cells were extracted with 0.5% Triton X-100 (Sigma, St Louis, MO, USA) in 20 mM HEPES, pH 7.4 (N[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]; Merck, Darmstadt, Germany), 50 mM NaCl, 1 mM EGTA (ethylene glycol bis(β-aminoethyl ether) N,N,N’,N’-tetraacetic acid; Sigma, St Louis, MO, USA), 1 mM PMSF (phenylmethylsulfonyl fluoride), 10 μg/ml leupeptin and 100 μM sodium orthovanadate (Sigma, St Louis, MO, USA). The pellets were washed in the same buffer and finally resuspended in 50 mM Tris-HCl, pH 7.2 (Tris; Sigma, St Louis, MO, USA) for analysis of PLC activity (Payrastre et al., 1991). The preparation of the different membrane fractions is described below.

PLC activity present in the different subcellular fractions was detected using [3H]phosphatidylinositol(4,5)bisphosphate (Amersham International, Amersham, UK) as exogenous substrate. [3H]phosphatidylinositol(4,5)bisphosphate (30,000 dpm, 1 nmol/assay) was dried under a N2 stream and dissolved in 5% sodium cholate; the reaction was performed at 30°C for 10 minutes as previously described (Rock and Jackowski, 1987).

Immunolabelling

EGF stimulation of A431 cells was stopped after 0, 2, 5 and 10 minutes of incubation with 100 ng/ml EGF by adding an equal volume of fixative, 4% formaldehyde in 100 mM PIPES (piperazine-N,N’-bis[2-ethanesulfonic acid]; Merck, Darmstadt, Germany; Griffiths, 1993). The mixture was immediately replaced by fresh fixative and fixation was continued for 30 minutes at room temperature. After washing with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4) cells were permeabilized for 5 minutes with 0.5% Triton X-100 in CSK (100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 10 mM PIPES, pH 6.8, 1.2 mM PMSF; Fey et al., 1986) followed by washing with CSK. To visualize PLC-γ1 and F-actin simultaneously the preparations were incubated with a mixture of monoclonal mouse antibodies against PLC-γ1 (5 μg/ml; Upstate Biotechnology Inc., Lake Placid, NY, USA) and tetramethylrhodamine isothiocyanate (TRITC) labelled phalloidin (1 μg/ml; Sigma, St Louis, MO, USA). For co-localization of PLC-γ1 and EGF-receptor or proteins phosphorylated on tyrosine the following antibodies were used: a polyclonal rabbit antibody (1 μg/ml) raised against a peptide which is unique to PLC-γ1 (sc-81; Santa Cruz, CA, USA) and a monoclonal anti-EGF-receptor antibody 2E9 (Defize et al., 1989) or anti-phosphotyrosine antibody (1 μg/ml; PY 20, ICN Biomedical Inc., Costa Mesa, CA, USA). The anti-PLC-γ1 antibodies are detected with secondary goat anti-mouse or anti-rabbit antibodies tagged with fluorescein isothiocyanate, the anti-EGF-receptor and anti-phosphotyrosine antibodies with goat anti-mouse antibodies tagged with Texas Red, both at a concentration of 7 μg/ml (Jackson Immunological Research Laboratory Inc. Avondale, PA, USA). All antibodies used for immunolabelling were diluted in PBS (pH 7.4) containing 0.05% cold water fish gelatin and 0.5% bovine serum albumin (both Sigma, St Louis, MO, USA). Light microscopic preparations were mounted on glass slides in Mowiol (Rodriguez and Deinhardt, 1960) supplemented with 0.1% p-phenylene diamine to reduce fading (Johnson and De C. Nogueira Araujo, 1981) and recorded with a confocal laser scanning microscope (Bio-Rad MRC 600, UK; Zeiss, Germany). Six to eight optical sections were recorded per sample (Figs 4, 6, 8). The intense PLC-γ1 labelling of the cytoplasm would mask the weaker signal at the membrane ruffles. Therefore the stacks of optical sections were split in two to depict the basal and the apical aspect of the cell separately.

Analysis of the membrane fraction

A431 cells grown in culture flasks (Costar, Cambridge, MA, USA)
were kept for 4 hours in serum-free medium at 37°C to reduce basal activity. Thereafter they were incubated without or with 100 ng/ml EGF for 30 minutes at 4°C. A fraction enriched in plasma membrane vesicles was prepared as described by Todderud et al. (1990; Cohen and Fava, 1985). The cells were harvested in HEPES buffer (20 mM HEPES, pH 7.4, 150 mM KCl, 2 mM MgCl₂, 4 mM benzamidine, 1 mM PMSF) by scraping the cells off the culture flask. After centrifugation at 600 g for 5 minutes at 4°C the pellet was resuspended in 0.5 ml HEPES buffer and forced 15 times through a 25 G needle. Intact cells and the cell nuclei were pelleted at 600 g for 5 minutes at 4°C. After 20 minutes centrifugation at 8,000 g (4°C) the fraction enriched in plasma membrane was recovered from the pellet, whereas the supernatant predominantly represented the cytosolic fraction. In order to determine whether the PLC-γ₁ binds to the membrane skeleton the vesicle fraction was further treated with 1% Triton X-100 in HEPES buffer and pelleted (12 minutes, 13,000 rpm; centrifuge 5415C, Eppendorf), the supernatant corresponds to the Triton-X soluble, i.e. membrane fraction and the pellet to the insoluble, i.e. membrane skeleton fraction.

The proteins were separated by SDS-PAGE in 10% acrylamide gels (Laemmli, 1970) and transferred electrophoretically to nitrocellulose (Towbin et al., 1979). The blots were incubated with 2 μg/ml anti-PLC-γ₁ antibodies (Upstate Biotechnology Inc., Lake Placid, NY, USA) and detected with alkaline phosphatase-coupled secondary antibodies (120 ng/ml; Jackson Immunological Research Laboratory Inc. Avondale, PA, USA) as described by Knecht and Dimond (1984) or with 1 μg/ml anti-phosphotyrosine antibodies (PY 20) and detected with horseradish peroxidase-coupled anti-mouse antibodies (80 ng/ml; Jackson Immunological Research Laboratory Inc. Avondale, PA, USA) followed by ECL detection (Amersham International, Amersham, UK).

The membrane fraction was further analysed by electron microscopy. The fraction was frozen between two copper platelets in a cryojet (JFD 030, Bal-Tec, Balzers, Lichtenstein) as described by Müller et al. (1980), fractured in a freeze-fracture device (BAF 300, a cryojet (JFD 030, Bal-Tec, Balzers, Lichtenstein) as described by Knecht and Dimond (1984) or with 120 ng/ml anti-mouse antibodies (80 ng/ml; Jackson Immunological Research Laboratory Inc. Avondale, PA, USA) and shadowed with 2 nm platinum/carbon (Moor, 1973).

RESULTS

Effect of EGF on the localization of phospholipase C-γ₁ in A431 cells

As described previously (Payrastre et al., 1991) and as shown in Table 1, PLC activity is present in cytoskeletal fractions of A431 cells. Treatment of intact A431 cells with EGF results in a 40% increase of PLC activity associated with the cytoskeleton (Table 1).

In order to gain an insight into the possible role of activation and association of PLC-γ₁ to the cytoskeleton by the activated EGF-receptor, the localization of PLC-γ₁ was studied by immunofluorescence in control and EGF treated cells (Fig. 1). PLC-γ₁ appears to be randomly distributed in the cytoplasm of untreated A431 cells (Fig. 1A). No labelling was observed in the cell nucleus. Treatment of A431 cells with EGF for two minutes at 37°C resulted in a redistribution of PLC-γ₁ to the plasma membrane area (Fig. 1B), in particular to the newly formed membrane ruffles.

In order to establish the possible interaction of the membrane-associated PLC-γ₁ with the cytoskeleton, A431 cells were fractionated into a cytosol and a membrane fraction. These experiments were done at 4°C to avoid large variations in time of stimulation due to the extended fractionation protocol. The membrane fraction consisted predomi-

Table 1. Effect of EGF on PLC activity in the cytoskeletons of A431 cells

<table>
<thead>
<tr>
<th>PLC activity (pmol min⁻¹ mg⁻¹)</th>
<th>Control</th>
<th>EGF</th>
</tr>
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<tr>
<td>Specific activity of PLC</td>
<td>7.0±2.3</td>
<td>10.0±2.9</td>
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</table>

A431 cells were treated with or without 200 ng EGF for 10 minutes at 37°C. Thereafter cytoskeletons were prepared and assayed for PLC activity. In a control sample containing bovine serum albumin less than 1 pmol min⁻¹ mg⁻¹ was detected. PLC activity is expressed in pmol inositol trisphosphate formed per minute and mg protein. The results are means ± s.e.m. from ten different experiments.

![Fig. 1](image_url) A431 cells either unstimulated (A) or stimulated for 2 minutes with 100 ng/ml EGF (B) at 37°C were labelled for PLC-γ₁ using a mixture of monoclonal antibodies. In unstimulated cells PLC-γ₁ is uniformly distributed in the cytoplasm. After 2 minutes of EGF stimulation ruffling of the plasma membrane and a distinct location of PLC-γ₁ at those ruffles is observed. Bar, 25 μm.
The data described above clearly demonstrate that treatment of A431 cells with EGF results in a translocation of PLC-γ1 from the cytoplasm to the plasma membrane (Figs 1B, 3A). In addition, it was demonstrated that EGF caused an increase in PLC activity associated with the cytoskeleton of A431 cells (Table 1). In order to establish whether these two phenomena, i.e. the localization of PLC-γ1 at the plasma membrane and association of PLC activity with the cytoskeleton, are related to each other, the membrane fraction was treated with Triton X-100 and separated by centrifugation into a Triton X-100 soluble fraction, i.e. the membranes, and a Triton X-100 insoluble fraction, i.e. the membrane skeleton. The PLC-γ1 present in the membrane fraction is associated mostly with the membranes (Fig. 3B, lane 1, 2) and to a lesser extent with the membrane skeleton (Fig. 3B, lane 3, 4). Treatment of the cells with EGF causes a translocation of PLC-γ1 to the membrane fraction (Fig. 3A, lane 4). Only a minor part of the PLC-γ1 appears to move to the membrane skeleton (Fig. 3B, lane 4) upon EGF stimulation. Identical fractions were also analyzed for PLC activity (Table 2). Upon EGF stimulation PLC activity increases 6.4-fold in the membrane fraction and 1.7-fold in the membrane skeleton fraction.

These data suggest that only a small fraction of the translocated PLC is associated with the membrane skeleton. To further investigate the relationship between PLC translocated to the plasma membrane and the cytoskeleton, we have studied the localization of PLC-γ1 and F-actin by confocal scanning laser microscopy. As shown in Fig. 1A, PLC-γ1 appears to be randomly distributed in the cytoplasm of untreated A431 cells. Double labelling of the cells with phalloidin to visualize the actin microfilaments clearly demonstrates that no co-localization is present with F-actin and PLC-γ1 in untreated A431 cells (Fig. 4, 0 minutes). Also no co-localization has been observed between PLC-γ1 and microtubules or intermediate filaments (data not shown). These observations indicate that only a minor fraction of the PLC-γ1 is associated with the cytoskeleton which accounts for the PLC activity measured on isolated cytoskeletons as shown in Table 1.

Exposure of A431 cells to EGF for 2, 5 or 10 minutes, respectively, results in a rapid reorganization of F-actin as described previously (Rijken et al., 1991). The actin reorganization is due to cortical actin polymerization and depoly-

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Cytosol</th>
<th>Membrane skeleton</th>
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<tbody>
<tr>
<td>Control</td>
<td>13.6±1</td>
<td>6.2±6</td>
</tr>
<tr>
<td>EGF</td>
<td>51.3±30</td>
<td>39.5±22</td>
</tr>
<tr>
<td></td>
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<td>102.6±18</td>
</tr>
</tbody>
</table>

After EGF treatment the cells were fractionated into cytosol, plasma membrane and membrane skeleton. The different fractions were assayed for PLC activity. PLC activity is expressed in pmol inositol trisphosphate formed per minute and per mg protein. The results are means ± s.e.m. from three different experiments.
Fig. 4. A431 cells either unstimulated (0 minutes) or stimulated for 2, 5 and 10 minutes with 100 ng/ml EGF at 37°C were labelled for PLC-γ1 using a mixture of monoclonal antibodies (PLC). F-actin is visualized with fluorescent phalloidin (actin). In unstimulated cells PLC-γ1 is uniformly distributed in the cytoplasm. After 2 minutes of EGF stimulation ruffling of the plasma membrane and a co-localization of PLC-γ1 with the microfilaments is observed at those ruffles (arrows). After 5 minutes PLC-γ1 is still associated with the membrane covering the former ruffles (arrowhead) but the strong co-localization with F-actin is lost. Bar, 25 μm.
merization of stress fibres. As shown in Fig. 4 after 2 to 5 minutes the actin polymerization is most pronounced in newly formed membrane ruffles (arrow). The ruffling disappears again after 10 minutes of EGF exposure and the cells start to round up (Fig. 4). Labelling the same cells for PLC-1 demonstrates that EGF causes a rapid translocation of PLC-1 from the cytosol to the plasma membrane, in particular to the membrane ruffles, where PLC-1 co-localizes with F-actin (Fig. 4, arrow). When the ruffles get smoothed (after around 5 minutes of stimulation) PLC-1 may still be associated with the membrane covering the area of the former ruffles (Fig. 4, arrowhead) but the strong co-localization to F-actin is lost. After 10 minutes of EGF treatment most of the membrane ruffles have disappeared and the PLC-1 has dissociated from the plasma membrane and almost no co-localization is observed between PLC-1 and F-actin (Fig. 4), which is in contrast with the earlier time points at 2 and 5 minutes of stimulation (Fig. 4). Thus in summary, these data demonstrate that EGF treatment of A431 cells results within 2 minutes in the formation of membrane ruffles. The formation of the ruffles is correlated with the EGF-induced reorganization of F-actin. A strong and transient co-localization between PLC-1 and F-actin is observed in the membrane ruffles.

**Co-localization of PLC-1 and the EGF-receptor**

It has been well established that PLC-1 is one of the substrates of the EGF-receptor tyrosine kinase (Meisenhelder et al., 1989). Binding of EGF results in the phosphorylation of the EGF-receptor; after 30 seconds the receptor is already phosphorylated (Fig. 5, lane 2) and in A431 cells it remains phosphorylated for up to 1 hour under EGF-stimulation (Sturani et al., 1988). The activated receptor in its turn phosphorylates PLC-1. Within 30 seconds of the addition of EGF PLC-1 is phosphorylated (Fig. 5, lane 2). Disappearance of the phosphotyrosine signal after 2 minutes of incubation with EGF (Fig. 5, lane 4) suggests that dephosphorylation also occurs rapidly. These results correlate with those published by Meisenhelder et al. (1989) on the phosphorylation of PLC-1 in platelet derived growth factor stimulated fibroblasts.

Phosphorylation of PLC-1 by the EGF-receptor tyrosine kinase requires a physical interaction of the two proteins which is mediated by the SH2 domains of PLC-1. Since EGF-induced translocation of PLC-1 to the membrane ruffles occurs in the same period of time as the PLC-1 phosphorylation, it is tempting to suggest that phosphorylation of PLC-1 occurs in the membrane ruffles. Therefore, we have studied a possible co-localization between PLC-1 and the EGF-receptor by double labelling using confocal microscopy. In untreated A431 cells no co-localization between EGF-receptor and PLC-1 is observed (Fig. 6, 0 minutes). PLC-1 is present predominantly in the cytoplasm, while the EGF-receptor resides at the plasma membranes mainly where they are in contact with neighbouring cells. If EGF is added to the cells PLC-1 translocates to the membrane ruffles within minutes. Within 2 to 5 minutes also the EGF-receptors move to the same sites (Fig. 6; arrow). After 10 minutes of EGF treatment the membrane ruffles disappear, PLC-1 disperses into the cytoplasm, EGF-receptors are internalized and co-localization disappears. These data suggest that the EGF-receptors form complexes with their substrates at the membrane ruffles.

**Membrane ruffles: the site of signal transduction**

The data described above suggest that the membrane ruffles formed upon activation of the cells by EGF constitute the site of signal transduction between the receptor and its substrates. To substantiate this hypothesis, we have studied the localization of proteins phosphorylated on tyrosine in A431 cells. The main fraction of phosphotyrosyl proteins is found in the membrane fraction (Fig. 7, lane 2) and only a minor part in the cytoplasm fraction (Fig. 7, lane 1). After treatment with Triton X-100 most of the proteins phosphorylated on tyrosine are solubilized (Fig. 7, lane 3) but a distinct number of proteins is also associated with the membrane skeleton (Fig. 7, lane 4). The most obvious of the membrane skeleton associated phosphotyrosyl proteins are the EGF-receptor and PLC-1. These results show that the proteins phosphorylated on tyrosine are mainly membrane associated. A number of the most prominent of the phosphotyrosyl proteins (e.g. EGF-receptor, PLC-1) are also associated with the underlying membrane skeleton.

Double labelling experiments with anti-PLC-1 and anti-phosphotyrosine antibodies show that untreated A431 cells contain hardly any tyrosine phosphorylated proteins (Fig. 8, 0 minutes). In some cells the focal contact sites were stained weakly. Treatment of the cells with EGF for various periods of time as indicated causes a rapid phosphorylation on tyrosine residues. After 2 minutes of EGF treatment, proteins phos-
Fig. 6. A431 cells either unstimulated (0 minutes) or stimulated for 2, 5 and 10 minutes with 100 ng/ml EGF at 37°C were labelled for PLC-γ1 with a polyclonal antibody raised against a peptide which is unique to PLC-γ1 (PLC). The EGF-receptor is visualized with a monoclonal antibody (EGFr). In unstimulated cells the EGF-receptor is localized predominately at the plasma membrane between neighbouring cells. After 2 minutes of EGF stimulation the EGF-receptor shows prominent co-localization with PLC-γ1 at the membrane ruffles (arrows). Later the co-localization disappears. Bar, 25 μm.
of EGF to the cells a clear co-localization between the receptor area on the plasma membrane. Within 2 minutes after addition receptor and one of its major substrates, PLC-\(\gamma\)-1 demonstrate a transient co-localization between the EGF approximately 10 minutes of stimulation. In this paper we membrane ruffles. These membrane ruffles are formed within membrane fraction. The position of PLC-\(\gamma\)-1 (PLC) was assessed by stripping the nitrocellulose and reprobing with an anti-PLC-\(\gamma\)-1 antibody (not shown). EGFr, EGF-receptor; PLC, PLC-\(\gamma\)-1. The relative molecular masses in kDa are indicated.

DISCUSSION

Treatment of A431 cells with EGF causes a drastic reorganization of the actin microfilament system, stress fibres disappear whereas actin polymerization is induced at the apical plasma membrane (Rijken et al., 1991). In addition the plasma membrane located actin polymerization has been implicated to be involved in the growth factor induced formation of membrane ruffles. These membrane ruffles are formed within minutes of growth factor addition and disappear again after approximately 10 minutes of stimulation. In this paper we demonstrate a transient co-localization between the EGF receptor and one of its major substrates, PLC-\(\gamma\)-1, at a specific area on the plasma membrane. Within 2 minutes after addition of EGF to the cells a clear co-localization between the receptor and PLC-\(\gamma\)-1 was observed especially in the newly formed membrane ruffles. Furthermore, using antibodies directed against phosphotyrosine residues, a strong co-localization was observed between the EGF receptor and PLC-\(\gamma\)-1 on the one hand and tyrosine phosphorylated proteins on the other. Also this co-localization was apparent at the membrane ruffles during 2 to 5 minutes after addition of EGF, but had already decreased after 10 minutes. Interestingly, the co-localization occurs at the same period of time after addition of EGF as the EGF induced phosphorylation and activation of PLC (Fig. 5; Meisenhelder et al., 1989; Liu and Carpenter, 1992). These observations strongly suggest that the membrane ruffles constitute the site of action of EGF induced signal transduction. This hypothesis is in line with a number of observations published previously. Thus it has been shown that various substrates of the EGF receptor, i.e. calpactin II, ezrin and spectrin, are translocated to the newly formed membrane ruffles after activation of the cells with EGF. This translocation was accompanied by tyrosine phosphorylation of these receptor substrates (Campos-Gonzalez et al., 1990; Bretscher, 1989), comparable to the translocation of PLC-\(\gamma\)-1 described in this paper. Furthermore, using immunofluorescence and electron microscopy studies, it has been demonstrated that the phosphorylated EGF receptor resides in the membrane ruffles (Carpentier et al., 1987).

An intriguing aspect of the notion that membrane ruffles represent the site of growth factor induced signal transduction across the plasma membrane, concerns the formation of the membrane ruffles in relation to the activation of the receptor tyrosine kinase and the growth factor induced actin polymerization. It is of interest to mention that the EGF receptor is an actin binding protein (den Hartigh et al., 1992). In addition it has been shown that in intact A431 cells not all EGF receptors are associated with the cytoskeleton, but only the high affinity class of receptors (Wiegant et al., 1986; van Bergen en Hengouwen et al., 1989). The high affinity class of receptors has been shown to be responsible for EGF-induced signal transduction (Defize et al., 1989). Recently, evidence has been obtained that the association of the EGF receptor to the cytoskeleton results in a potentiation of the receptor tyrosine kinase (Gronowski and Bertsics, 1993). Thus exposure of cells to EGF will result in a rapid binding of EGF to the cytoskeleton-associated, high affinity class receptors, and in the activation of the tyrosine kinase. The activated receptor tyrosine kinase will subsequently phosphorylate its substrates, probably especially those substrates that reside in the neighbourhood of the receptor. Amongst these substrates also PLC-\(\gamma\)-1 will be phosphorylated and activated. The activated PLC-\(\gamma\)-1 will convert in its turn phosphatidylinositol(4,5)bisphosphate (PIP\(_2\)) into inositol triphosphate (IP\(_3\)) and diacylglycerol (DG) in the inner leaflet of the plasma membrane. According to the bilayer-couple theory (Sheetz and Singer, 1974), the plasma membrane will form a bleb or ruffle directed towards the medium, because of the fact that DG possesses less surface than PIP\(_2\) and because DG is uncharged in contrast to PIP\(_2\). Therefore the action of PLC causes a decrease in surface tension of the inner leaflet of the membrane (Demel et al., 1975; James et al., 1994). Furthermore, evidence has been obtained that DG stimulates the formation of actin nucleation sites at the plasma membrane (Shariff and Luna, 1992), and consequently plasma membrane associated actin polymerization becomes possible.
A431 cells either unstimulated (0 minutes) or stimulated for 2, 5 and 10 minutes with 100 ng/ml EGF at 37°C were labelled for PLC-γ1 with a polyclonal antibody raised against a peptide which is unique to PLC-γ1 (PLC). The proteins phosphorylated on tyrosine were visualized with an anti-phosphotyrosine antibody (PY). In unstimulated cells hardly any proteins are phosphorylated on tyrosine. After 2 minutes of EGF stimulation most of the phosphotyrosyl proteins are found in the membrane ruffles together with PLC-γ1 (arrows). Bar, 25 μm.

Fig. 8.
This actin polymerization in turn is necessary to stabilize the newly formed membrane ruffles.

Another aspect of interest concerns the role of actin in the membrane ruffle located signal transduction. As outlined above, actin polymerization may be involved in the formation of the membrane ruffles, but in addition actin may also play a role in signal transduction by serving as a matrix for the components involved in the signal transduction cascade. The EGF receptor itself has been demonstrated to be an actin binding protein (den Hartigh et al., 1992), but in addition it has been shown that also the SH3 domain of PLC is able to bind to actin fibres (Bar-Sagi et al., 1993). Interestingly, other proteins such as GRB2 are targeted through their SH3 domain to the membrane ruffles (Bar-Sagi et al., 1993). These results further substantiate the hypothesis that the membrane ruffles form an ‘organelle’ to initiate intracellular signalling.

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