Silencing of the epidermal growth factor receptor in the absence of the ligand requires phospholipase C activity

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Abstract The possible involvement of phospholipase Cβ (PLCβ) in a crosstalk mechanism between G-protein coupled receptors and receptor tyrosine kinases was investigated in HeLa-S3 and A-431 cells. A basic activity of the receptor for epidermal growth factor (EGF) in the absence of its ligand was found only in A-431 cells overexpressing this receptor. Inhibition of PLC drastically increased EGF receptor activity in both cell lines, suggesting that PLC activity is necessary for the silencing of the EGF receptor in the absence of its ligand. Activation of PLCβ and protein kinase C (PKC) via G-protein-linked ATP receptors greatly diminished the basic EGF receptor activity in A-431 cells. This negative regulation was prevented by the protein tyrosine phosphatase inhibitor, vanadate. The results suggest a crosstalk between a G-protein-linked receptor and a receptor tyrosine kinase, involving signalling via PLCβ and PKC to a downstream protein tyrosine phosphatase functioning in the control of EGF receptor activity.

Key words: Receptor crosstalk; Phospholipase C; Protein tyrosine phosphatase; G-protein-coupled receptor; Receptor tyrosine kinase

1. Introduction

Signal transduction is the biological language used for cell–cell communication in a multicellular organism [1,2]. Because target cells are usually addressed by multiple signalling molecules simultaneously, there is a need for signal sorting and integration within the cell. Different signalling pathways should be able to communicate with each other, a phenomenon termed receptor crosstalk or transmodulation [3,4]. Understanding the mechanisms by which diverse signalling cascades interact with each other is fundamental to an understanding of how the process of signal transduction is regulated in vivo.

Transmodulation of signalling pathways has been studied with growth factor receptors that possess intrinsic tyrosine kinase activity (RTKs), e.g. the receptors for epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). Binding of PDGF to its receptor leads to the phosphorylation of the EGF receptor on threonine 654 [3,4]. This phosphorylation converts high affinity to low affinity EGF receptors. The effect of PDGF is similar, but not identical to the one produced by activation of PKC with phorbol esters. In HeLa cells, PDGF acts as a dominant inhibitor of signalling via high affinity EGF receptors [5]. Mitogen activated protein kinase (MAP) has also been shown to negatively regulate the EGF receptor by phosphorylating threonine-669 [6]. Recently, it was suggested that threonine phosphorylation of the EGF receptor is not sufficient for negative regulation of its kinase activity, but that protein tyrosine phosphatases (PTP) activated by PKC and MAP kinase may be involved [7,8].

Activation of PKC and subsequent transmodulation of the EGF receptor requires diacylglycerol (DAG) and calcium ions. Production of these activators of PKC is facilitated by phosphatidylinositol specific phospholipases C [9]. Two major pathways lead to the activation of phospholipase C (PLC) [9,10]: one is the receptor tyrosine kinase (RTK) pathway, exemplified by the PDGF receptor. Activation of the PDGF receptor causes dimerization and autophosphorylation on tyrosine residues. The tyrosine phosphorylated residues – in their specific amino acid sequence surroundings – represent high affinity binding sites for SH_2-domain containing proteins (src homology domain 2), such as PLCγ, Src kinase, and PI3 kinase [11]. After binding to the receptor, PLCγ becomes tyrosine phosphorylated and activated. PLCγ then cleaves the lipid precursor phosphatidylinositol 4,5-bisphosphate to give both DAG and inositol-trisphosphate (IP3). IP3 causes mobilization of intracellular calcium, which together with DAG leads to the activation of PKC. The second pathway is initiated by the activation of G-protein-coupled 'serpentine' receptors, having 7 transmembrane domains [9,10]. Binding of a ligand to these receptors causes dissociation of the heterotrimeric G-protein and subsequent activation of PLCβ by the G_a subunit. PLCβ catalyses the same reaction as PLCγ. The common theme in both of these signalling pathways appears to be activation of PKC through the action of PLC species. We therefore investigated whether G-protein-activated PLCβ is involved in the transmodulation of RTK activity. It is shown here that PLCβ activity is necessary to silence the EGF receptor in the absence of the ligand. This inactivation probably involves a protein tyrosine phosphatase that is coupled to G-protein-linked receptors via PLCβ and PKC.

2. Materials and methods

The human cell lines HeLa-S3 and A-431 were obtained from the American Type Culture Collection. Minimum Essential Medium (MEM) was from BioWhittaker. Horse serum was from Boehringer-Mannheim. Epidermal growth factor and the PLC inhibitor substance U73122 were from Biomol; tetraacetate-phorbol-13-myristate (TPA = PMA) and adenosine triphosphate (ATP) were from Sigma.

HeLa cells and A-431 cells were routinely grown in MEM/10% horse serum. For experiments, they were seeded into 24-well culture vessels ($2–3 \times 10^4$ per well) and cultured for 3–4 days. The medium was then replaced with fresh medium supplemented with 0.5% horse serum. After additional 24 h, cells were treated for 5 min with EGF (final concentration 1 or 50 nM) dissolved in PBS, with TPA dissolved in DMSO (final concentration 2 μM), or with ATP dissolved in PBS and neutralized to pH 7.0 (final concentration 150 μM). Sodium vanadate (final concentration 100 μM) was added one hour before the respective

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factors. PLC was inhibited by the addition of U73122 dissolved in DMSO (final concentration 30 μM) for 40 min. As a control, cells were treated with either PBS and/or with DMSO. The medium was aspirated and cells were lysed in 150 μl of electrophoresis sample buffer. Samples were applied to 7% SDS-PAGE. Immunoblotting was performed using an anti-phosphotyrosine antibody (PY-20) conjugated with horseradish peroxidase (ICN), followed by enhanced chemiluminescence detection (ECL from Amersham).

3. Results

The intrinsic tyrosine kinase activity of the EGF receptor is activated upon ligand binding and is responsible for the autophosphorylation of the receptor and the subsequent phosphorylation of substrate proteins on tyrosine residues. Immunoblotting of cell lysates with anti-phosphotyrosine antibodies was used to assess this activity.

Phosphorylation of the EGF receptor on tyrosine residues was not detectable in HeLa cells that were serum-starved for 24 h, e.g. in the absence of EGF. After treatment with the phospholipase C inhibitor U73122, a tyrosine phosphorylated protein of M_r 160 kDa was observable (Fig. 1), corresponding to the autophosphorylated EGF receptor as verified by immunoprecipitation with an anti-EGF receptor antibody (not shown).

Human squamous carcinoma cells A-431 overexpress the EGF receptor. Serum-starvation for 24 h greatly diminished the amount of autophosphorylated EGF receptors (Fig. 1). However, a basic amount of tyrosine phosphorylation was still detectable. As in HeLa cells, inhibition of PLC caused increased tyrosine phosphorylation of the EGF receptor in A-431 cells (Fig. 1). Stimulation of A-431 cells with EGF caused strong phosphorylation of the 160 kDa protein, corroborating its identity as the EGF receptor. In addition, tyrosine phosphorylation of other cellular proteins was also visible. The pattern of tyrosine phosphorylated cellular proteins after EGF treatment was not identical but quite similar to the one observed after inhibition of PLC. These data indicate that inhibition of PLC causes activation of the EGF receptor tyrosine kinase in the absence of its ligand.

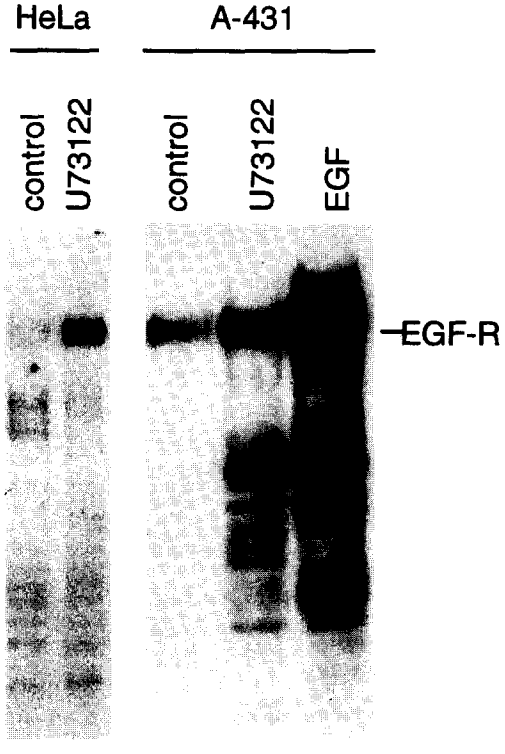

Fig. 1. Tyrosine phosphorylated proteins in HeLa cells and A-431 cells after treatment with PLC inhibitor. Cells were serum-starved for 24 h. The PLC inhibitor U73122 (dissolved in DMSO) was added for 40 min and EGF (50 nM) for 5 min. Control cells were treated with DMSO. Cells were lysed and tyrosine phosphorylated proteins were detected by immunoblotting using anti-phosphotyrosine antibodies (PY-20) conjugated with horseradish peroxidase, and subsequent ECL development. The position of the EGF receptor is indicated as EGF-R.

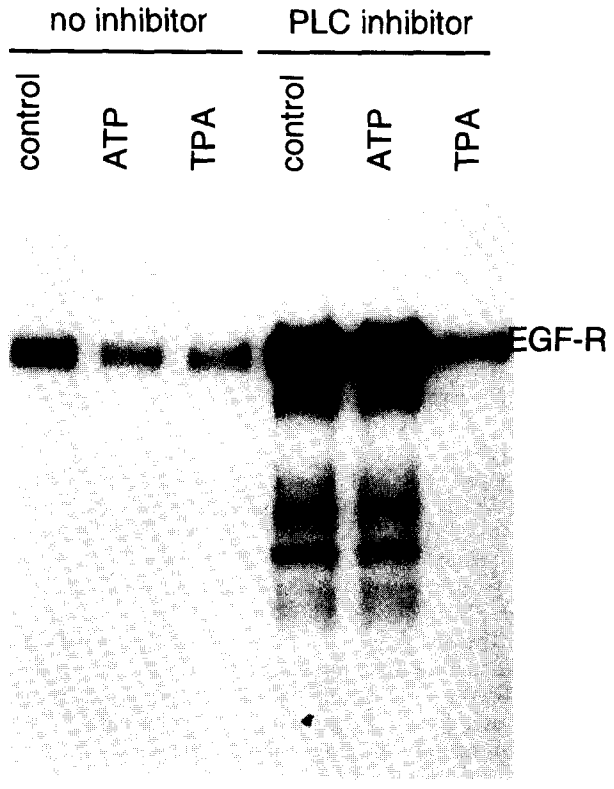

Fig. 2. Downregulation of EGF receptor activity in A-431 cells treated with ATP and TPA. Serum-starved cells were incubated with DMSO or with the PLC inhibitor U73122 in DMSO for 40 min followed by stimulation with ATP and TPA for 5 min. Control cells received DMSO alone. Cells were lysed and tyrosine phosphorylated proteins were detected as described in legend to Fig. 1. The position of the EGF receptor is indicated as EGF-R.

If inhibition of PLC increased EGF receptor activity, then activation of PLC should have the opposite effect. The presence of a basic EGF receptor activity in serum-starved A-431 cells permits one to investigate the effect of PLC activators on EGF receptor autophosphorylation. PLCβ species are activated by G-protein-coupled seven transmembrane domain ('serpentine') receptors [9,10]. Extracellular ATP is an agonist of G-protein-linked P2-purinergic receptors in A-431 cells [12]. It is shown in Fig. 2 that ATP treatment greatly diminished the amount of tyrosine phosphorylated EGF receptors in serum-starved cells, corroborating that activation of PLCβ leads to an inactivation of the EGF receptor tyrosine kinase. This inactivation was prevented in cells that were pretreated with PLC inhibitor (Fig. 2), confirming that transmodulation of the EGF receptor after ATP treatment occurred via activation of PLCβ species.

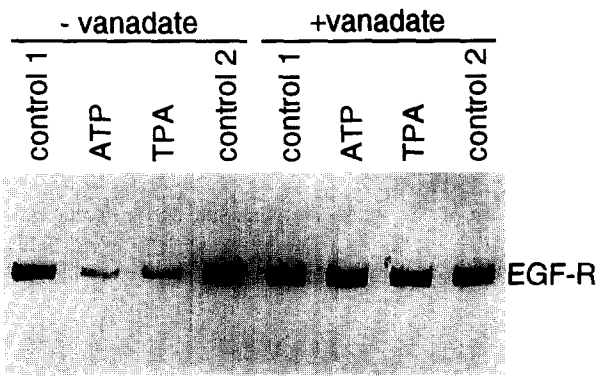

Fig. 3. Requirement of protein tyrosine phosphatase activity for silencing of the EGF receptor (EGF-R). Serum-starved cells were incubated with PBS (−vanadate) or with sodium vanadate dissolved in PBS (+vanadate) for 1 h, followed by stimulation with ATP or TPA for 5 min. Control cells received DMSO (control 1) or PBS (control 2). Cells were lysed and tyrosine phosphorylated proteins were detected as described in legend to Fig. 1.

PLC liberates diacylglycerol (DAG) from its membrane lipid precursor. DAG is an activator of PKC. In cultured cells, this kinase can also be activated by the structurally related phorbol ester, TPA. It is shown in Fig. 2 that treatment of A-431 cells with TPA decreased autophosphorylation of the EGF receptor. This inactivation was not prevented by the PLC inhibitor.

It was suggested recently that inhibition of the ligand-induced signalling by the EGF receptor may involve a protein tyrosine phosphatase (PTP) downstream of PKC [7]. Serum-starved A-431 cells were therefore preincubated with the PTP inhibitor, vanadate, followed by stimulation with ATP and TPA. This experiment revealed that silencing of the EGF receptor after treatment with ATP and TPA does not occur when tyrosine phosphatases are inhibited (Figs. 3,4).

Activation of PKC by TPA or PDGF has been shown to cause inhibition of the ligand induced signalling by the EGF receptor [3]. A-431 cells were therefore treated with EGF and ATP simultaneously. Tyrosine phosphorylation of the EGF receptor increased rapidly after addition of EGF alone, and reached a maximal level after 20 min. In the presence of ATP, however, EGF-induced autophosphorylation was markedly reduced, suggesting that activation of PLCβ by G-protein-coupled ATP receptors is inhibitory for signalling by the EGF receptor also in the presence of the ligand, EGF.

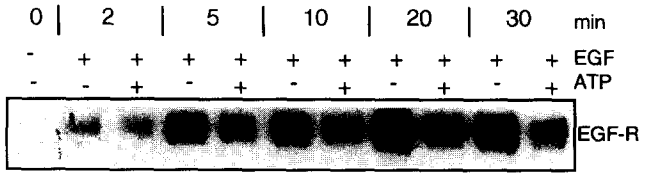

Fig. 4. Inhibition of EGF-induced autophosphorylation of the EGF receptor (EGF-R) in the presence of ATP. Serum-starved A-431 cells were stimulated with EGF (1 nM) alone ore with EGF and ATP (150 μM) simultaneously. At the indicated times after stimulation, cells were lysed and tyrosine phosphorylated proteins were detected as described in legend to Fig. 1. The basic level of EGF receptor phosphorylation before stimulation is not visible in this case because of the short exposure time during ECL development.

4. Discussion

Growth factor receptors with intrinsic tyrosine kinase activity are considered to enter an inactive state in the absence of the ligand. Binding of the ligand induces dimerization and autophosphorylation of the receptors on tyrosine residues [11], followed by tyrosine phosphorylation of substrate proteins. Protein tyrosine phosphatases counteract the kinases and remove phosphate from signalling molecules [13]. Here we showed that the EGF receptor is active and produces a signal even in the absence of EGF when PLC species are inhibited. This suggests that deprivation of the ligand by serum-starvation is not sufficient to retain the EGF receptor in an inactive state. Instead, a basic activity of PLC species and, hence, a continuous inhibitory signalling cascade is responsible and necessary for a complete silencing of the EGF receptor in the absence of the ligand.

In serum-starved A-431 cells, the basic PLC activity apparently is not sufficient to inactivate all EGF receptors. This is easily explained by the presence of large amounts of receptors due to an overexpression of the respective gene [14]. However, after ATP treatment and subsequent activation of PLCβ, the residual EGF receptor activity was greatly diminished. This suggests that not only PLCγ, but also PLCβ is involved in the transmodulation of signalling pathways and in the heterolo-

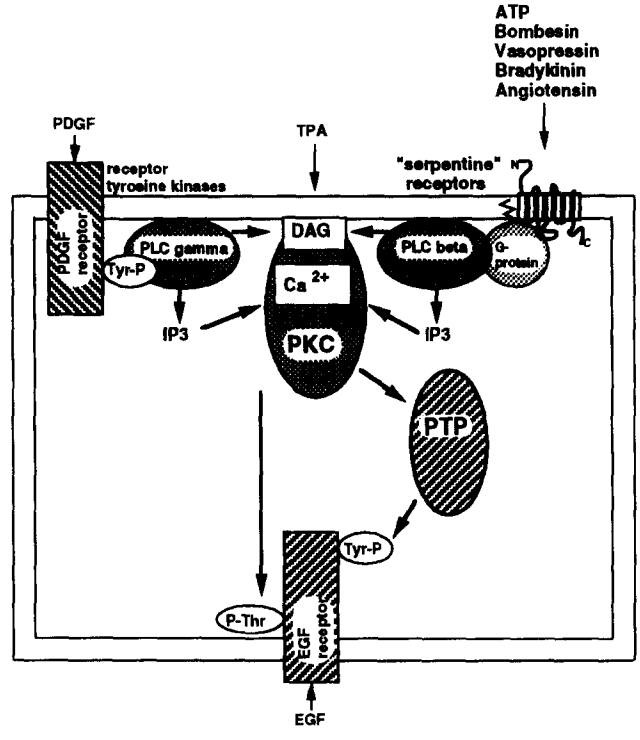

Fig. 5. Proteins involved in the transmodulation of the EGF receptor by other receptor tyrosine kinases and G-protein-linked receptors. Upon binding of the ligand, G-protein coupled 'serpentine' receptors activate PLC gamma, and RTKs (PDGF receptor) PLCβ. Both of these pathways increase the diacylglycerol (DAG) and calcium ion concentration, resulting in activation of protein kinase C (PKC). PKC activation results in the phosphorylation of the EGF receptor at threonine residues, converting high affinity to low affinity receptors. Concomitantly, a protein tyrosine phosphatase (PTP) is activated downstream of PKC, which dephosphorylates tyrosine residues on the EGF receptor.

gous regulation of RTK activity. Apparently, activation of G-protein-coupled 'serpentine' receptors such as P2 purinergic ATP receptors produces a signal that influences EGF receptor activity, suggesting a crosstalk between signalling cascades of G-protein-linked receptors and RTK-mediated signalling.

Activation of PLCβ after binding of extracellular ATP to P2y or P2u receptors is well documented [12]. In accordance with this, inhibitors of PLC prevented silencing of the EGF receptor by ATP treatment. However, inactivation of the EGF receptor was achieved with TPA even in the presence of PLC inhibitor. This suggests that ATP exerts its effect on the EGF receptor via PLCβ-produced DAG and subsequent activation of PKC. Dephosphorylation of the EGF receptor after ATP and TPA treatment was sensitive to the protein tyrosine phosphatase inhibitor, vanadate, suggesting that the activity of such a phosphatase is required for silencing of the EGF receptor in the absence of a ligand, and that this PTP lies downstream of PKC.

Transmodulation of the EGF receptor after PDGF treatment has been assessed by determining the conversion of high affinity to low affinity EGF binding sites, resulting from activation of the PLCγ/PKC pathway and threonine phosphorylation of the EGF receptor [3,4]. This transmodulation therefore should not be sensitive to the PTP inhibitor, vanadate. Here we showed that activation of PKC with phorbolester does not decrease autophosphorylation of the EGF receptor when PTPs are inhibited, suggesting that threonine phosphorylation is not responsible for inhibition of the EGF receptor activity. Instead, silencing of the EGF receptor in the absence of the ligand requires permanent activation of a protein tyrosine phosphatase via the PLC/PKC pathway.

Interestingly, activation of PLC has been shown for all of the known ligands of G-protein-coupled receptors, including those for bombesine [15], vasopressin [16], angiotensin, histamine, acetylcholine, thromboxane A_2, bradykinin [9], ATP [12], and thrombin [17]. In addition, some of these ligands, including ATP, have been shown to convert high affinity to low affinity EGF receptors, involving a PKC dependent mechanism [15,16,18]. Therefore, activation of PKC and transmodulation of the EGF receptor by phosphorylation on threonine residues, as well as the subsequent activation of an EGF receptor specific protein tyrosine phosphatase, are likely to represent components of a crosstalk mechanism common to all of these ligands of G-protein-coupled receptors and also to those RTKs that activate PLCγ (Fig. 5).

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