Published in final edited form as:

Pflugers Arch. 2011 March ; 461(3): 387–397. doi:10.1007/s00424-010-0904-3.

Phosphatidylinositol-4,5-bisphosphate regulates epidermal growth factor receptor activation

Ioannis E. Michailidis,

Department of Structural and Chemical Biology, Mount Sinai School of Medicine, New York, NY, USA

Radda Rusinova,

Department of Structural and Chemical Biology, Mount Sinai School of Medicine, New York, NY, USA

Anastasios Georgakopoulos,

Department of Psychiatry, Mount Sinai School of Medicine, New York, NY, USA

Yibang Chen,

Department of Pharmacology and Systems Therapeutics, Mount Sinai School of Medicine, New York, NY, USA

Ravi Iyengar,

Department of Pharmacology and Systems Therapeutics, Mount Sinai School of Medicine, New York, NY, USA

Nikolaos K. Robakis,

Department of Psychiatry, Mount Sinai School of Medicine, New York, NY, USA

Diomedes E. Logothetis, and

Department of Physiology and Biophysics, Virginia Commonwealth University School of Medicine, 1101 E Marshall St, Richmond, VA 23298, USA

Lia Baki

Department of Physiology and Biophysics, Virginia Commonwealth University School of Medicine, 1101 E Marshall St, Richmond, VA 23298, USA

Abstract

Phosphatidylinositol-4,5-bisphosphate [PI(4,5)P₂ or PIP₂] is a direct modulator of a diverse array of proteins in eukaryotic cells. The functional integrity of transmembrane proteins, such as ion channels and transporters, is critically dependent on specific interactions with PIP₂ and other phosphoinositides. Here, we report a novel requirement for PIP₂ in the activation of the epidermal growth factor receptor (EGFR). Down-regulation of PIP₂ levels either via pharmacological inhibition of PI kinase activity, or via manipulation of the levels of the lipid kinase PIP5K1 α and the lipid phosphatase synaptojanin, reduced EGFR tyrosine phosphorylation, whereas up-regulation of PIP₂ levels via overexpression of PIP5K1 α had the opposite effect. A cluster of

© Springer-Verlag 2010

delogothetis@vcu.edu; albaki@vcu.edu.

Electronic supplementary material The online version of this article (doi:10.1007/s00424-010-0904-3) contains supplementary material, which is available to authorised users.

Present Address: I. E. Michailidis Department of Biological Sciences, Columbia University, New York, NY 10027, USA

Present Address: R. Rusinova Department of Physiology and Biophysics, Weill Medical College of Cornell University, New York, NY 10065, USA

positively charged residues in the juxtamembrane domain (basic JD) of EGFR is likely to mediate binding of EGFR to PIP₂ and PIP₂-dependent regulation of EGFR activation. A peptide mimicking the EGFR juxtamembrane domain that was assayed by surface plasmon resonance displayed strong binding to PIP₂. Neutralization of positively charged amino acids abolished EGFR/PIP₂ interaction in the context of this peptide and down-regulated epidermal growth factor (EGF)-induced EGFR autophosphorylation and EGF-induced EGFR signaling to ion channels in the context of the full-length receptor. These results suggest that EGFR activation and downstream signaling depend on interactions of EGFR with PIP₂ and point to the basic JD's critical involvement in these interactions. The addition of this very different class of membrane proteins to ion channels and transporters suggests that PIP₂ may serve as a general modulator of the activity of many diverse eukaryotic transmembrane proteins through their basic JDs.

Keywords

PIP₂; EGF receptor; Phosphorylation; Signaling; Plasma membrane

Introduction

Phosphatidylinositol-4,5-bisphosphate [PI(4,5)P₂ (or PIP₂)] is a phospholipid of pivotal importance to eukaryotic membranes. Receptor-induced hydrolysis of PIP₂ by phospholipase C (PLC) into the second messengers diacylglycerol and inositol triphosphate and receptor-mediated phosphorylation of PIP₂ by phosphatidylinositol 3-kinase (PI3K) into PIP₃ initiate two vital receptor-regulated signaling pathways (the PLC and PI3K pathways, respectively), whereas PIP₂ itself can bind to an increasing number of effectors, influencing a variety of cellular processes [5, 7]. PIP₂ has been implicated in cytoskeletal organization [14, 51], membrane trafficking and vesicle exocytosis, and endocytosis and motility [24, 47]. Decreased nerve terminal PIP₂ levels lead to synaptic defects and early postnatal lethality in mice [6]. Most ion channels and transporters tested show dependence of their activities on levels of plasma membrane PIP₂ [42]. Examples of PIP₂-dependent ion channels include K⁺ channels, such as the inward rectifiers (Kir) [18] and the M-current [43, 52], voltage-gated Ca²⁺ channels [49], TRP channels [3, 35], and NMDA receptors [23, 26] (for a recent review, see Logothetis et al. [20]). Extracellular signals acting through G protein-coupled or growth factor receptors that can cause PIP₂ hydrolysis, dramatically affect transporter function and ion channel gating [42]. Structure-function studies aiming to identify the channel sites of interaction with PIP₂ have revealed a hotspot of interactions in a highly basic region of the juxtamembrane domain (JD) of the protein immediately proximal to the membrane.

The epidermal growth factor receptor (EGFR, also known as ErbB1) is a member of the receptor tyrosine kinase (RTK) family. The EGFR and other ErbB RTKs play crucial roles in cell growth, survival, proliferation, and differentiation as well as carcinogenesis. Mice lacking EGFR die shortly after birth due to multi-organ failure, developing skin, lung, pancreas, and gastrointestinal tract abnormalities and progressive neurodegeneration [27, 40]. Binding of EGF induces dimerization of EGFR monomers, followed by trans-autophosphorylation of key tyrosine residues on the EGFR intracellular tails, which up-regulates their intrinsic RTK activity [38]. The EGFR is functionally coupled to the turnover of PIP₂, as direct tyrosine phosphorylation of phospholipase C γ by activated EGFR triggers PIP₂ hydrolysis to IP₃ and diacylglycerol, which leads to Ca²⁺ mobilization and protein kinase C (PKC) activation, respectively [32, 33].

Although studies of effects of EGF on excitable membranes are expanding, the mechanism that links EGF stimulation to the trans-autophosphorylation and activation of the receptor

remains elusive. It is however increasingly appreciated that the JD of EGFR plays a central role in the activation process [1, 15, 25, 31, 44]. Recent structural studies coupled with mutagenesis have provided compelling evidence that the distal JD (termed JM-B or JMAD) stabilizes the activated dimer via interactions revealed by crystal structures [25, 44]. Yet, the precise role of the proximal JD (here referred to as the basic JD) is not clear [44]. Here we use a variety of approaches (i.e., biochemical, genetic, and electrophysiological) to show that EGFR phosphorylation and downstream signaling to ion channels depend on receptor interactions with PIP₂. A cluster of positively charged residues in the basic JD of EGFR is likely to be involved in the binding of EGFR to PIP₂. Neutralization mutations of the positively charged amino acids abolishes EGFR/PIP₂ interaction, suppresses EGF-induced EGFR auto-phosphorylation and down-regulates EGF-induced EGFR signaling to ion channels. Furthermore, pharmacological or genetic down-regulation of PIP₂ levels decreases EGF-induced receptor phosphorylation, whereas up-regulation of PIP₂ levels augments it. Since EGFR activation leads to hydrolysis of PIP₂, we propose that its functional dependence on PIP₂ can serve as a mechanism of self-desensitization.

Materials and methods

Chemicals, antibodies

Wortmannin was purchased from Sigma and antibodies were purchased from Cell Signaling Technologies (EGFR_{P-Y992}), Santa Cruz Biotechnologies (EGFR, β -tubulin), Millipore (anti phospho-Tyrosine 4 G10) and Genetex (PIP5KI α).

Surface plasmon resonance

A Biacore 2000 optical biosensor was used together with Biacore streptavidin-coated (SA) sensor chips and BIA Evaluation software. The SA chips were washed and equilibrated in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) or phosphate-buffered saline (PBS) buffer (pH 8.0, 20 μ l/min), Soluble biotinylated C6 PIP₂ was purchased from Echelon and 1–10 mM stocks were prepared in water. After brief sonication, biotinylated PIP₂ was immobilized by consecutive injections (3 μ l/min, 90 min) until an approximate increase of 300 resonance units was achieved. Peptides were introduced at five serial dilutions in Biacore buffer (1 mM DTT, 2.5 mM MgCl₂, 20 mM HEPES, 1 mM EDTA, 150 mM NaCl, 0.005% P20) in 60–100 μ l injection volumes and at a flow rate of 20 μ l/min. Binding was measured at 25°C for 5 min, followed by 15 min of dissociation. Then 1 min of wash with regeneration buffer was performed (1 M NaCl, 12.5 mM NaOH). The whole process was then repeated three times for each dilution sample. Sensorgram data were analyzed with the BIA evaluation kinetics package and the equilibrium affinity constant was calculated. Peptides were at least 95% pure (Biosynthesis).

cDNAs, mutagenesis, cRNAs

Human EGFR, Kir3.4*(S143T) (the Kir3.4 mutant that yields large homomeric currents), synaptojanin (a gift from Dr. Pietro DeCamilli, Yale University, CT) and PI(5)P kinase (a gift from Drs. Show-Ling Shyng and Colin Nichols, Washington University, MO) cDNAs were subcloned into the high-expression vector pGEMsH [34]. Point mutations were introduced using the Quikchange mutagenesis kit (Stratagene) and custom primers with nucleotide mismatch (Invitrogen) and were confirmed by sequencing. cRNAs were transcribed using an in vitro transcription kit with the T7 promoter (Ambion).

Oocyte preparation and injections

Stage V or VI *Xenopus laevis* oocytes were surgically removed from ovaries and digested with collagenase using standard methods [19]. Oocytes were kept for 2–4 days at 17°C.

Oocytes were injected (50 nl/oocyte) with full-length wild-type or mutant EGFR (5 ng), Kir3.4*(S143T) (2 ng), synaptojanin (10 ng) or PI(5)P kinase (10 ng) cRNAs, and analyzed 2–3 days post injection.

Electrophysiological recordings

Electrophysiological recordings were performed 2–4 days after injection using a DAGAN two-electrode voltage-clamp amplifier and currents were obtained with a square pulse protocol. For the measurements of calcium-activated chloride currents, the bath solution (Ringer's) contained (in mM): 115 NaCl, 2.5 KCl, 1.8 BaCl₂, and 10 HEPES, pH 7.2. For the experiments with Kir3.4 (we used the S143T mutant that yields large homomeric currents) the bath solution (high potassium, HK) contained (in mM): 91 KCl, 1 NaCl, 1 MgCl₂, and 5 HEPES/KOH. HK supplied with 3–4 mM BaCl₂ was added at the end of the experiment to block potassium currents and obtain a measurement of the leak current. Rapid solution exchange was achieved with a gravity flow perfusion system converging on a 6 µl oocyte chamber engineered in our laboratory from inert materials.

Western immunoblotting

Equal numbers of 25–50 oocytes for each experimental group were stimulated with EGF (100 ng/ml for 10 min) in PBS supplied with phosphatase inhibitors (10 mM NaVO₄, 10 mM NaF) and directly homogenized in 5 mM Tris–HCl pH 8.0, 1 mM EDTA, 1 mM EGTA containing 10 mM NaVO₄, 10 mM NaF and complete protease inhibitor cocktail (Roche). For preparation of the membrane fraction, homogenates were centrifuged for 5 min at 5,000 RPM (4°C) and supernatants were subsequently ultracentrifuged at 100,000×g for 40 min at 4°C in a Beckman Maxima Ultracentrifuge. The pellets were re-suspended with a suitable volume of lysis buffer (0.5–1 µl per oocyte), mixed with sodium dodecyl sulfate (SDS)-PAGE loading buffer, boiled for 10 min and analyzed by Western blotting. Total EGFR levels in the samples were determined either on the same membrane used for phosphorylated EGFR (after stripping and re-probing) or on duplicate membranes. For the statistical analysis, densitometric data were normalized for EGFR expression levels and expressed relative to the normalized value of the control in each experiment. Results are expressed as mean±SEM. Statistical significance was determined with a Student's *t* test.

HeLa cell and siRNA experiments

HeLa cells were plated in 60-mm dishes and serum starved with 0.5% fetal bovine serum for 16 h in the presence of 100 nM or 15 µM Wortmannin, or vehicle DMSO. After 16 h, the cells were stimulated with 100 ng/ml EGF for 10 min, then lysed in 1%SDS solubilization buffer in the presence of protease (Roche) and phosphatase (Sigma) inhibitor cocktails. 50 µg of lysate was analyzed by Western immunoblotting (WB) as described above. For siRNA transfection, HeLa cells were plated and transfected according to the manufacturer's (NeoFx kit, Ambion) instructions, using siRNA targeting exons 9,10 of human PIP5KIα in a final concentration of 100 nM in OPTIMEM medium. Cells were incubated for 48–72 h before assaying.

Results

Mutations of EGFR closest to the membrane interface suppress its auto-phosphorylation and downstream signaling

McLaughlin et al. [25] used fluorescence resonance energy transfer to show that a peptide made of the EGFR JD could sequester PIP₂. Using surface plasmon resonance (SPR) as the assay to confirm EGFR/PIP₂ interaction, we reproduced these results, showing that a peptide mimicking the EGFR juxtamembrane domain (region 645–662) displayed strong binding to

PIP₂ (Fig. 1b, top panel). Inspection of the JD of EGFR, as well as other RTKs, shows the presence of a cluster of positively charged residues that alternate with hydrophobic residues (Fig. 1a). The pattern of basic and hydrophobic residues is reminiscent of the basic JDs of most ion channels [8, 13, 21, 35, 52], as well as the PIP₂-binding motifs of some cytoskeletal proteins [51]. Neutralization of the first eight positively charged amino acids abolished EGFR/PIP₂ interaction in the SPR assay, further highlighting the importance of the eight positively charged amino acids within the 645–662 region for EGFR/PIP₂ interaction (Fig. 1b, middle panel). Interestingly, this positively charged region of the EGFR juxtamembrane domain has been shown to be of critical importance for EGFR activation. Deletion of the amino acids between 645 and 657 of the EGFR JD region has been shown to abolish receptor dimerization and autophosphorylation [1], while a mutant EGFR bearing the same eight mutations as the ones used in our SPR experiments (Fig. 1b, middle panel) has previously been shown to lack tyrosine phosphorylation when heterologously expressed [50]. In view of these earlier reports, our data raise the possibility that EGFR activation and downstream signaling depend on receptor interaction with PIP₂. To address this possibility, we first aimed at assessing the role of PIP₂-interacting basic residues of the EGFR JD on receptor auto-phosphorylation, using a system appropriate for subsequent physiological studies. *X. laevis* oocytes provide a heterologous expression system, where ion channel function can be conveniently studied and where the EGFR has been shown to reconstitute with similar properties as in mammalian cells [29]. We used this system to express full length wild-type or mutant EGFR bearing Arg to Asn substitutions within the PIP₂ binding domain (see Fig. 2a). An anti total EGFR antibody was used to determine levels of expression of wild-type and mutant EGFR (see Fig. 2b for its specificity), whereas an antibody that specifically recognizes phosphorylated tyrosine 992 of the EGFR (EGFR_{Y992}) was used to determine basal and EGF-induced phosphorylation of wild-type and mutant EGFR (Fig. 2c–e). Figure 2c shows that phosphorylation at Y992 of the mutant EGFR bearing the eight Arg to Asn substitutions (ASN8) is clearly impaired, indicating that the same amino acids that are critical for the receptor/PIP₂ interaction are also critical for its auto-phosphorylation. From the other two mutants, ASN3 appeared to have the highest impact. Similar results were obtained using a general anti-phosphotyrosine antibody (4 G10; Fig. 2d). Quantification of the data on the EGF-induced EGFR phosphorylation at Y992 from several independent experiments ($n=6-8$) revealed that the ASN3 mutation was almost as potent as the ASN8 in suppressing EGF-induced Y992 phosphorylation, while mutation of the five distal Arg residues (ASN5) resulted in a weaker, but statistically significant suppression of receptor phosphorylation (Fig. 2e).

To exclude the possibility that the impaired EGFR phosphorylation seen with the ASN3 and ASN8 mutants was due to a decrease in plasma membrane localization, we compared surface expression levels of wild-type EGFR with those of EGFR bearing the ASN3 and ASN8 mutations. Online Resource Figure S1 shows that the mutations did not interfere with the surface expression of the receptor, in good agreement with previous reports showing that neither neutralization [50], nor deletion [1] of the 645–657 region of EGFR prevented its expression at the cell surface. Together, our data indicate that neutralization of the basic amino acids between 645 and 657 residues of the EGFR JD region (a) suppressed its autophosphorylation and (b) the three most proximal to the membrane Arg residues (ASN3 or R645–R647) are the most critical for receptor phosphorylation. In agreement with these data, alanine scanning mutagenesis in a recent study by Red Brewer et al. [31] in 2009 revealed R646 and R647 as the two most critical residues in the entire 645–657 region for EGFR phosphorylation.

To investigate the effect of the above JD mutations on EGF-dependent downstream signaling, we asked whether the mutations affect EGF-dependent regulation of ion channel activity. *Xenopus* oocytes possess endogenous outwardly rectifying (i.e., conduct more

outward than inward current at similar electrochemical driving forces) Ca^{2+} -activated chloride (CaCl) channels that are activated by the Ca^{2+} released from IP_3 -dependent Ca^{2+} stores [46]. Electrophysiological monitoring of CaCl currents (I_{CaCl}) in *Xenopus* oocytes provides a sensitive assay of intracellular Ca^{2+} and can therefore serve as a native sensor of Ca^{2+} release (an alternative to Ca^{2+} -sensitive dyes) that follows stimulation of PLC-coupled receptors (e.g., EGFR, M1 ACh receptors, etc.). Figure 3 shows that oocytes expressing wild-type or mutant EGFR could all elicit at positive potentials outward I_{CaCl} when stimulated by EGF (Fig. 3a, upper traces), thus ensuring that the mutant EGFR could still signal downstream.

We have previously shown that PIP_2 hydrolysis through EGFR signaling inhibits the activity of both heteromeric Kir3.1/3.4 and homomeric Kir3.4* (Kir3.4-S143T) inwardly rectifying channels [17]. Inhibition of Kir3 currents provides better quantification of the relative effect of the EGFR mutations on EGF-dependent inhibition than I_{CaCl} , possibly due to the desensitizing character of I_{CaCl} . Thus, we used oocytes co-expressing Kir3.4* together with either wild-type or mutant EGFR. Figure 3b shows that GIRK4* currents prior to inhibition by EGF were at comparable levels of expression regardless of whether they were co-expressed with wild-type or mutant EGFR. Simultaneous monitoring of outwardly and inwardly rectifying currents enabled us to examine whether EGFR signaling took place (outward I_{CaCl}), while at the same time to quantify the extent of the signaling (inhibition of inward Kir3.4* currents). Figure 3a (lower traces) and (summary of data) show that the ASN5 mutant inhibited Kir3.4* currents upon EGF stimulation almost as effectively as the wild-type EGFR. In contrast, the ASN8 and ASN3 mutants, although they were still functional in eliciting I_{CaCl} , were significantly less effective in inhibiting Kir3.4* currents upon EGF stimulation (Fig. 3a, c).

Together, these data show that mutation of the first three Arg residues in the PIP_2 -binding region of the EGFR basic JD is enough to suppress receptor autophosphorylation and downstream signaling to ion channels, in complete agreement with the alanine scanning mutagenesis data of Red Brewer et al. [31], reporting R646 and R647 as the two most critical residues in the entire 645–657 region for EGFR phosphorylation. Unlike the above mentioned group, however, we were not able to further narrow down the critical region, as each of the two point mutations that we tested (R645N and R646N) did not result in significant differences in EGF-induced Kir3 current inhibition when compared to wild-type EGFR (data not shown).

Functional control of EGFR phosphorylation by altering PIP_2 levels

To investigate whether PIP_2 can modulate EGFR function, we used the PI kinase inhibitor Wortmannin. Generally, Wortmannin inhibits PI(4) kinase (PI4K) at micromolar concentrations, in addition to blocking PI(3) kinase (PI3K), at nanomolar concentrations [28]. PI-4 K is a critical enzyme in the synthetic cascade of PIP_2 that catalyzes the addition of phosphate to the 4' position of the inositol ring of PI, leading to the formation of the PIP_2 precursor PI(4)P [45]. Online resource Figure S2 shows that treatment of oocytes with 15 μM of Wortmannin for 1 h reduced PIP_2 levels to about 40% of control (see Online resource Figure S2). Thus, in agreement with previously published data [48], Wortmannin can effectively reduce PIP_2 levels. Figure 4a shows a robust, progressive decrease in the EGF-induced $\text{EGFR}_{\text{Y992}}$ phosphorylation elicited by increasing concentrations of Wortmannin treatment. Wortmannin treatment (100 nM or 15 μM for one hour) reduced $\text{EGFR}_{\text{Y992}}$ phosphorylation three- and sixfold, respectively (Fig. 4b), whereas the amounts of EGFR present, probed with an anti-total EGFR antibody were not significantly different. A similar inhibition of EGFR phosphorylation was observed when we probed phosphotyrosine at position 1045 (data not shown). Since low concentrations of Wortmannin mainly affect PI-3 K rather than PI4K, the threefold reduction observed with 100 nM of Wortmannin implies a

possible contribution of PIP₃. This may be specific to the oocyte system, since 100 nM of Wortmannin was ineffective in suppressing EGFR phosphorylation in HeLa cells (see Fig. 5a, b). Even in oocytes however, the additional reduction of EGFR phosphorylation observed upon treatment with 15 μ M of Wortmannin (a concentration that reduces PIP₂ levels to about 40% of control, see Online Resource Figure S2), suggests that EGFR activation depends on PIP₂. Consistent with this hypothesis, earlier work has shown that both PI4K and PIP5K interact with the EGFR [4].

To further test our hypothesis, we employed synaptojanin, a protein that has also been shown to interact with the EGFR, and whose constitutive PIP₂ 5-phosphatase activity hydrolyzes PIP₂ to PI(4)P [36]. Introduction of synaptojanin mRNA in oocytes decreased EGFR_{Y992} phosphorylation (Fig. 4c, d), further supporting the correlation between decreased PIP₂ and inhibition of EGFR phosphorylation. If decreases in PIP₂ levels result in diminished EGFR phosphorylation, then an increase in PIP₂ levels would be expected to have the opposite effect. Indeed, overexpression of PIP5KI α (a final enzyme in the pathway that leads to PIP₂ formation), produced a significant gain in the phosphorylation of wild-type EGFR (Fig. 4e, f). Importantly, phosphorylation of the ASN8 mutant (which does not bind PIP₂, see Fig. 1, EGFR_{645-662MUT}) was not affected by PI(5)PKI α overexpression (Fig. 4e, f, see ASN8 $\pm$ PIP5KI α). These findings further support the notion that EGFR activation depends on the levels of PIP₂.

To test whether the observed effect of PIP₂ on autophosphorylation of heterologously expressed EGFR also applies to mammalian cells with native EGFRs, we used HeLa cells, a cell line of mammalian origin that expresses EGFR endogenously. Figure 5a and b show that EGF-induced phosphorylation of Y992 in the endogenous EGFR of HeLa cells was not significantly affected by 100 nM Wortmannin (overnight application in parallel with serum starvation), but was significantly inhibited by 15- μ M Wortmannin.

RNA interference was used to down-regulate the levels of the lipid kinase PIP5KI α , in order to suppress production of PIP₂. This treatment indeed led to a significant reduction in the levels of PIP5KI α (Fig. 5c, d), whereas levels of β -tubulin were not affected (see Fig. 5c). Interestingly, HeLa cells transfected with the siRNA for PIP5KI α displayed a marked reduction in both their basal (which was high enough in three out of four batches of cells to allow detection by WB), and EGF-induced EGFR_{Y992} phosphorylation (Fig. 5e and f). The suppression of basal EGFR phosphorylation by PIP5KI α siRNA implies that spontaneous autophosphorylation of the receptor may also be sensitive to PIP₂. Alternatively, despite the overnight starvation, depletion of growth factors may not be complete and therefore what is referred to as basal phosphorylation most probably reflects, at least in part, the contribution of residual EGF-induced phosphorylation, which is sensitive to PIP₂.

Since it has been reported that it is the PIP5KI β and not the PIP5KI α isoform that is involved in EGFR internalization, it is unlikely that the siRNA-mediated reduction of the PIP5KI α isoform led to a decrease in EGFR autophosphorylation due to a decreased cell surface receptor density. To explicitly control for such a possibility, we subjected HeLa cells to FACS analysis to determine the cell surface receptor density before and after treatment with siRNA for the PIP5KI α isoform. EGF stimulation indeed caused a reduction in the cell surface receptor density, as has been previously shown [41]. However, treatment with siRNA for the PIP5KI α isoform had no effect on cell surface receptor density before or after EGF treatment (see Online resource Figure S3). Thus, the effect of the siRNA of the PIP5KI α isoform on autophosphorylation of EGFR was not due to changes in receptor internalization.

Discussion

Phosphoinositides, and in particular phosphatidylinositol (4,5)-bisphosphate, are emerging as central regulators of diverse cellular functions through their effects on transmembrane or plasma membrane associated proteins. Among the best examples of PIP₂-sensitive proteins are a long list of ion channels, the activity of which depends on their association with PIP₂ [18, 42], and the myristoylated alanine-rich C kinase substrate (MARCKS), which acts as a PIP₂ buffer to regulate the availability of PIP₂ [10, 30]. Data from the present study place EGFR among the PIP₂-dependent/PIP₂-associated proteins. Pharmacological or genetic down-regulation of PIP₂ levels severely impaired EGFR activation, as measured by receptor auto-phosphorylation, whereas up-regulation of PIP₂ potentiated EGF-mediated activation of the receptor.

Our data suggest that specific positively charged residues at the N terminus of the basic juxtamembrane domain of EGFR (645–657) are responsible for receptor interaction with PIP₂. Mutation of positively charged residues to neutral ones abolished EGFR/PIP₂ interactions and drastically down-regulated receptor activation and signaling to ion channels. Further mutations helped us narrow the critical region to the first three residues of the basic cluster (R645, R646, and R647) as the most critical for receptor activation. In agreement with this conclusion, alanine scanning mutagenesis in a recent study by Red Brewer et al. [31] revealed R646A and R647A as the two most potent mutations in the entire 645–657 region in terms of suppressing EGFR phosphorylation. With regards to downstream signaling however, although the ASN3 mutation (R645N+R646N+R647N) in our study abolished EGFR-mediated inhibition of Kir3.4* currents, the R645N and R646N single point mutants were not effective.

Several previous reports have highlighted the importance of the EGFR juxtamembrane domain and in particular the 645–657 JM region, on the regulation of receptor activation. Both positive and negative regulatory roles on EGFR activation have been attributed to this region. The inhibitory effects have been attributed to PKC-mediated phosphorylation of Thr-654 at the juxtamembrane region of the EGFR [22, 37], whereas the entire 645–657 region appears to be indispensable for EGFR activation. Deletion of this region has been shown to result in loss of receptor dimerization and trans-autophosphorylation [1, 15, 44], whereas neutralization of the positively charged residue (Arg/Lys to Asn) mutations (the exact same mutations that abolish PIP₂ binding, see Fig. 1), severely down-regulated receptor phosphorylation in both NIH3T3 and NR6 cells [50]. Our data suggest that the PIP₂-interacting characteristic of the 645–657 region is key to the indispensable role of this region for EGFR activation.

Association of the JM domain of EGFR with PIP₂ has previously been shown [25] and it has been interpreted as a mechanism serving to keep the receptor in an inactive state, when not bound to the ligand [25]. According to the proposed model, upon ligand binding, and receptor dimerization, spontaneous dissociation of a small fraction of the receptors from the membrane could lead to a low level auto-phosphorylation, activation of PLC, IP₃-mediated calcium release with a subsequent recruitment of Ca/CaM to the JM domain of the EGFR. This Ca/CaM binding on EGFR would reverse the charge of the juxtamembrane domain, resulting in disengagement of the whole cytoplasmic domain of the receptor from the membrane and exposing the Receptor Tyrosine Kinase domain to phosphorylation and full activation of the receptor. Such a model predicts that prevention of EGFR/PIP₂ interaction would favor ligand-independent activation of EGFR and potentiate its EGF-mediated activation [25]. However, as discussed in more detail in [39], despite this prediction, mutations in the juxtamembrane domain of EGFR that prevent PIP₂ binding inactivate the receptor ([50] and the current study). Since the proposed calmodulin-dependent EGFR

regulation requires some level of receptor activation and downstream Ca^{2+} release [25], it is reasonable to hypothesize that this may be occurring at a step downstream of the PIP_2 -dependent regulation that was revealed in the present study.

The precise mechanism of action of PIP_2 on the EGFR is unknown. A crystal structure of the EGFR kinase domain by Zhang et al. [53] shows the formation of an asymmetric dimer between receptor monomers in the activated state. Even though the structure does not include the JD, the authors state that the positioning, flexibility and possible movements of the JD are important, especially since there seems to be a gap between the ends of the dimeric extracellular region (~20 Å apart) and the N-terminal residues of the two kinase monomers (~50 Å apart) [53]. A recent crystal structure by Red Brewer et al. [31], which includes the JD, confirms the asymmetric dimer between receptor monomers and nearly all of the donor acceptor interactions seen in the previous structure. Similar were the conclusions from another recent study by Jura et al. [15] who again show that the membrane distal segment of the juxtamembrane region of the receiver/acceptor kinase makes stabilizing contacts with the C-terminal lobe of the activator/donor kinase and propose a model according to which, the membrane-proximal segment of the juxtamembrane region of EGFR (645–663) forms an antiparallel helical dimer that engages the transmembrane helices of the activated receptor to facilitate formation of the asymmetric kinase dimer. The impairment in EGFR activation by the mutations employed in our study most likely reflects both inhibition of PIP_2 binding and prevention of asymmetric contact formation. The proposal by Jura et al. activating JD latch resembles the case of erbB2 in which, the transmembrane domains are involved in a rotational sliding of the two monomeric helices against each other to regulate dimerization [9]. Because the JD connects the kinase lobes to the transmembrane segments, such facts point to the JD, which can bind PIP_2 , as an important regulator of EGFR activation. Thus, the EGFR JD with the aid of PIP_2 may have a role similar to the JD segments of the FLT3 receptor tyrosine kinase, i.e., to correctly align and maintain proper register during transition states [11].

It is well established that receptor-induced PLC activation and PIP_2 hydrolysis underlie receptor-mediated inhibition of ion channel activity [16, 17, 52]. Spatiotemporal regulation of PIP_2 levels may serve to provide the cell with the appropriate flexibility to fine-tune certain signaling pathways. For example although PIP_2 is required for NMDA receptor activity [26], NMDAR-mediated PLC hydrolysis of PIP_2 is necessary for spine remodeling and AMPAR internalization during NMDA receptor-dependent LTD [12] and mice lacking $\text{PLC}\beta 1$, the predominant PLC isoform in the forebrain, are LTD deficient [2]. In the case of EGFR, in view of our data showing its dependence on PIP_2 for full activation and transmission of EGF-dependent signal, the EGFR-dependent PLC activation and PIP_2 hydrolysis may serve as a negative feedback loop to promote receptor inactivation after transmission of the signal to downstream effectors. This auto-inhibitory loop would serve to terminate EGFR-mediated activation of PLC, thus preventing over-depletion of PIP_2 due to sustained PLC activity. Consequently, the newly formed PIP_2 would be allowed to establish the appropriate environment for a new cycle of EGFR activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a National Institutes of Health grant (HL-59949) to DEL. Support for RI and YC was provided by NIH grant DK-38761. We would like to thank Heikki Vaananen, Sophia Gruszecki, Samantha Lee, and Dr. Mei Zhang for excellent technical assistance, Drs. Pietro DeCamilli (Yale University, CT) for the gift of synaptojanin, and Drs. Show-Ling Shyng and Colin Nichols (Oregon Health and Science University, OR, USA and

Washington University, MO, USA) for the gift of PI(5)P kinase. We thank Dr. Qi Zhao for help with the analysis of the FACS data. We would also like to thank Drs. Giorgos Panayotou (Fleming Institute, Athens, Greece), Mitchell Goldfarb (Hunter College, NY, USA), Michael Ehlers (Duke Neurobiology, NC, USA), Julia Sable (Columbia University, NY, USA), Tibor Rohacs (UMDNJ), Stuart Aaronson (Department of Oncological Sciences, MSSM) and the Logothetis laboratory members for insightful discussions and comments on the manuscript.

References

1. Aifa S, Aydin J, Nordvall G, Lundstrom I, Svensson SP, Hermanson O. A basic peptide within the juxtamembrane region is required for EGF receptor dimerization. *Exp Cell Res*. 2005; 302:108–114. [PubMed: 15541730]
2. Choi SY, Chang J, Jiang B, Seol GH, Min SS, Han JS, Shin HS, Gallagher M, Kirkwood A. Multiple receptors coupled to phospholipase C gate long-term depression in visual cortex. *J Neurosci*. 2005; 25:11433–11443. [PubMed: 16339037]
3. Chuang HH, Prescott ED, Kong H, Shields S, Jordt SE, Basbaum AI, Chao MV, Julius D. Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4, 5)P₂-mediated inhibition. *Nature*. 2001; 411:957–962. [PubMed: 11418861]
4. Cochet C, Filhol O, Payrastré B, Hunter T, Gill GN. Interaction between the epidermal growth factor receptor and phosphoinositide kinases. *J Biol Chem*. 1991; 266:637–644. [PubMed: 1845983]
5. Czech MP. PIP₂ and PIP₃: complex roles at the cell surface. *Cell*. 2000; 100:603–606. [PubMed: 10761925]
6. Di Paolo G, Moskowitz HS, Gipson K, Wenk MR, Voronov S, Obayashi M, Flavell R, Fitzsimonds RM, Ryan TA, De Camilli P. Impaired PtdIns(4, 5)P₂ synthesis in nerve terminals produces defects in synaptic vesicle trafficking. *Nature*. 2004; 431:415–422. [PubMed: 15386003]
7. Downes CP, Gray A, Lucocq JM. Probing phosphoinositide functions in signaling and membrane trafficking. *Trends Cell Biol*. 2005; 15:259–268. [PubMed: 15866030]
8. Fan Z, Makielski JC. Anionic phospholipids activate ATP-sensitive potassium channels. *J Biol Chem*. 1997; 272:5388–5395. [PubMed: 9038137]
9. Fleishman SJ, Schlessinger J, Ben-Tal N. A putative molecular-activation switch in the transmembrane domain of erbB2. *Proc Natl Acad Sci USA*. 2002; 99:15937–15940. [PubMed: 12461170]
10. Gamper N, Shapiro MS. Regulation of ion transport proteins by membrane phosphoinositides. *Nat Rev Neurosci*. 2007; 8:921–934.
11. Griffith J, Black J, Faerman C, Swenson L, Wynn M, Lu F, Lippke J, Saxena K. The structural basis for auto-inhibition of FLT3 by the juxtamembrane domain. *Mol Cell*. 2004; 13:169–178. [PubMed: 14759363]
12. Horne EA, Dell'Acqua ML. Phospholipase C is required for changes in postsynaptic structure and function associated with NMDA receptor-dependent long-term depression. *J Neurosci*. 2007; 27:3523–3534. [PubMed: 17392468]
13. Huang CL, Feng S, Hilgemann DW. Direct activation of inward rectifier potassium channels by PIP₂ and its stabilization by Gbetagamma. *Nature*. 1998; 391:803–806. [PubMed: 9486652]
14. Janmey PA, Lindberg U. Cytoskeletal regulation: rich in lipids. *Nat Rev Mol Cell Biol*. 2004; 5:658–666. [PubMed: 15366709]
15. Jura N, Endres NF, Engel K, Deindl S, Das R, Lamers MH, Wemmer DE, Zhang X, Kuriyan J. Mechanism for activation of the EGF receptor catalytic domain by the juxtamembrane segment. *Cell*. 2009; 137:1293–1307. [PubMed: 19563760]
16. Keselman I, Fribourg M, Felsenfeld DP, Logothetis DE. Mechanism of PLC-mediated Kir3 current inhibition. *Channels (Austin)*. 2007; 1:113–123. [PubMed: 18690019]
17. Kobrinsky E, Mirshahi T, Zhang H, Jin T, Logothetis DE. Receptor-mediated hydrolysis of plasma membrane messenger PIP₂ leads to K⁺-current desensitization. *Nat Cell Biol*. 2000; 2:507–514. [PubMed: 10934471]
18. Logothetis DE, Jin T, Lupyan D, Rosenhouse-Dantsker A. Phosphoinositide-mediated gating of inwardly rectifying K(+) channels. *Pflugers Archiv*. 2007; 455:83–95. [PubMed: 17520276]

19. Logothetis DE, Movahedi S, Satler C, Lindpaintner K, Nadal-Ginard B. Incremental reductions of positive charge within the S4 region of a voltage-gated K⁺ channel result in corresponding decreases in gating charge. *Neuron*. 1992; 8:531–540. [PubMed: 1550676]
20. Logothetis DE, Petrou VI, Adney SK, Mahajan R. Channelopathies linked to plasma membrane phosphoinositides. *Pflugers Arch*. 2010; 460:321–341. [PubMed: 20396900]
21. Lopes CM, Zhang H, Rohacs T, Jin T, Yang J, Logothetis DE. Alterations in conserved Kir channel-PIP2 interactions underlie channelopathies. *Neuron*. 2002; 34:933–944. [PubMed: 12086641]
22. Lund KA, Lazar CS, Chen WS, Walsh BJ, Welsh JB, Herbst JJ, Walton GM, Rosenfeld MG, Gill GN, Wiley HS. Phosphorylation of the epidermal growth factor receptor at threonine 654 inhibits ligand-induced internalization and down-regulation. *J Biol Chem*. 1990; 265:20517–20523. [PubMed: 2173710]
23. Mandal M, Yan Z. Phosphatidylinositol (4, 5)-bisphosphate regulation of N-methyl-D-aspartate receptor channels in cortical neurons. *Mol Pharmacol*. 2009; 76:1349–1359. [PubMed: 19770351]
24. Martin TF. PI(4, 5)P₂ regulation of surface membrane traffic. *Curr Opin Cell Biol*. 2001; 13:493–499. [PubMed: 11454457]
25. McLaughlin S, Smith SO, Hayman MJ, Murray D. An electrostatic engine model for autoinhibition and activation of the epidermal growth factor receptor (EGFR/ErbB) family. *J Gen Physiol*. 2005; 126:41–53. [PubMed: 15955874]
26. Michailidis IE, Helton TD, Petrou VI, Mirshahi T, Ehlers MD, Logothetis DE. Phosphatidylinositol-4, 5-bisphosphate regulates NMDA receptor activity through alpha-actinin. *J Neurosci*. 2007; 27:5523–5532. [PubMed: 17507574]
27. Miettinen PJ, Berger JE, Meneses J, Phung Y, Pedersen RA, Werb Z, Derynck R. Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. *Nature*. 1995; 376:337–341. [PubMed: 7630400]
28. Nakanishi S, Catt KJ, Balla T. A Wortmannin-sensitive phosphatidylinositol 4-kinase that regulates hormone-sensitive pools of inositolphospholipids. *Proc Natl Acad Sci USA*. 1995; 92:5317–5321. [PubMed: 7777504]
29. Opresko LK, Wiley HS. Functional reconstitution of the human epidermal growth factor receptor system in *Xenopus* oocytes. *J Cell Biol*. 1990; 111:1661–1671. [PubMed: 1976639]
30. Rauch ME, Ferguson CG, Prestwich GD, Cafiso DS. Myristoylated alanine-rich C kinase substrate (MARCKS) sequesters spin-labeled phosphatidylinositol 4, 5-bisphosphate in lipid bilayers. *J Biol Chem*. 2002; 277:14068–14076. [PubMed: 11825894]
31. Red Brewer M, Choi SH, Alvarado D, Moravcevic K, Pozzi A, Lemmon MA, Carpenter G. The juxtamembrane region of the EGF receptor functions as an activation domain. *Mol Cell*. 2009; 34:641–651. [PubMed: 19560417]
32. Rhee SG. Regulation of phosphoinositide-specific phospholipase C. *Annu Rev Biochem*. 2001; 70:281–312. [PubMed: 11395409]
33. Rhee SG, Choi KD. Multiple forms of phospholipase C isozymes and their activation mechanisms. *Adv Second Messenger Phosphoprotein Res*. 1992; 26:35–61. [PubMed: 1419362]
34. Rohacs T, Lopes CM, Jin T, Ramdya PP, Molnar Z, Logothetis DE. Specificity of activation by phosphoinositides determines lipid regulation of Kir channels. *Proc Natl Acad Sci USA*. 2003; 100:745–750. [PubMed: 12525701]
35. Rohacs T, Lopes CM, Michailidis I, Logothetis DE. PI(4, 5)P₂ regulates the activation and desensitization of TRPM8 channels through the TRP domain. *Nat Neurosci*. 2005; 8:626–634. [PubMed: 15852009]
36. Sakisaka T, Itoh T, Miura K, Takenawa T. Phosphatidylinositol 4, 5-bisphosphate phosphatase regulates the rearrangement of actin filaments. *Mol Cell Biol*. 1997; 17:3841–3849. [PubMed: 9199318]
37. Santiskulvong C, Rozengurt E. Protein kinase Cα mediates feedback inhibition of EGF receptor transactivation induced by Gq-coupled receptor agonists. *Cell Signal*. 2007; 19:1348–1357. [PubMed: 17307332]
38. Schlessinger J. Ligand-induced, receptor-mediated dimerization and activation of EGF receptor. *Cell*. 2002; 110:669–672. [PubMed: 12297041]

39. Sengupta P, Bosis E, Nachliel E, Gutman M, Smith SO, Mihalyne G, Zaitseva I, McLaughlin S. EGFR juxtamembrane domain, membranes, and calmodulin: kinetics of their interaction. *Biophys J*. 2009; 96:4887–4895. [PubMed: 19527647]
40. Sibilia M, Steinbach JP, Stingl L, Aguzzi A, Wagner EF. A strain-independent postnatal neurodegeneration in mice lacking the EGF receptor. *EMBO J*. 1998; 17:719–731. [PubMed: 9450997]
41. Sorkin A, Carpenter G. Interaction of activated EGF receptors with coated pit adaptins. *Science*. 1993; 261:612–615. [PubMed: 8342026]
42. Suh BC, Hille B. Regulation of ion channels by phosphatidylinositol 4, 5-bisphosphate. *Curr Opin Neurobiol*. 2005; 15:370–378. [PubMed: 15922587]
43. Suh BC, Hille B. Regulation of KCNQ channels by manipulation of phosphoinositides. *J Physiol*. 2007; 582:911–916. [PubMed: 17412763]
44. Thiel KW, Carpenter G. Epidermal growth factor receptor juxtamembrane region regulates allosteric tyrosine kinase activation. *Proc Natl Acad Sci USA*. 2007; 104:19238–19243. [PubMed: 18042729]
45. Toker A. The synthesis and cellular roles of phosphatidylinositol 4, 5-bisphosphate. *Curr Opin Cell Biol*. 1998; 10:254–261. [PubMed: 9561850]
46. Weber W. Ion currents of *Xenopus laevis* oocytes: state of the art. *Biochim Biophys Acta*. 1999; 1421:213–233. [PubMed: 10518693]
47. Wenk MR, De Camilli P. Protein-lipid interactions and phosphoinositide metabolism in membrane traffic: insights from vesicle recycling in nerve terminals. *Proc Natl Acad Sci USA*. 2004; 101:8262–8269. [PubMed: 15146067]
48. Willars GB, Nahorski SR, Challiss RA. Differential regulation of muscarinic acetylcholine receptor-sensitive polyphosphoinositide pools and consequences for signaling in human neuroblastoma cells. *J Biol Chem*. 1998; 273:5037–5046. [PubMed: 9478953]
49. Wu L, Bauer CS, Zhen XG, Xie C, Yang J. Dual regulation of voltage-gated calcium channels by PtdIns(4, 5)P₂. *Nature*. 2002; 419:947–952. [PubMed: 12410316]
50. Yamane K, Toyoshima C, Nishimura S. Ligand-induced functions of the epidermal growth factor receptor require the positively charged region asymmetrically distributed across plasma membrane. *Biochem Biophys Res Commun*. 1992; 184:1301–1310. [PubMed: 1317170]
51. Yin HL, Janmey PA. Phosphoinositide regulation of the actin cytoskeleton. *Annu Rev Physiol*. 2003; 65:761–789. [PubMed: 12471164]
52. Zhang H, Craciun LC, Mirshahi T, Rohacs T, Lopes CM, Jin T, Logothetis DE. PIP(2) activates KCNQ channels, and its hydrolysis underlies receptor-mediated inhibition of M currents. *Neuron*. 2003; 37:963–975. [PubMed: 12670425]
53. Zhang X, Gureasko J, Shen K, Cole PA, Kuriyan J. An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. *Cell*. 2006; 125:1137–1149. [PubMed: 16777603]

Fig. 1.

Neutralization of basic residues in the EGFR juxtamembrane domain (JD) abolishes binding to PIP₂. **a** Alignment of the JD domains of several RTKs reveals the highly basic nature of these domains. **b** The sensorgrams shown were obtained by application (on/off times indicated by the arrows) of the respective peptides on the surface of a Biacore streptavidin-coated sensor chip, where a C6, biotinylated PIP₂ had been immobilized. *Upper panel* EGFR₆₄₅₋₆₆₂, *middle panel* EGFR_{645-662MUT}, *lower panel* a peptide derived from residues 1156–1172 of the distal C-terminus of the EGFR (EGFR₁₁₅₆₋₁₁₇₂) lacking clusters of positively charged residues. Sensorgrams are representative of multiple trials on three different sensor chips, with identical results. Peptide concentrations were (nM): for EGFR₆₄₅₋₆₇₂ 4,000, 2,000, 1,000, 500, and 250, for EGFR_{645-672MUT} 500, 250, 125, 62.5, and 31.125, for EGFR₁₁₅₆₋₁₁₇₂ 2,000, 1,000, 500, 250, and 125. Sensorgrams obtained from EGFR₆₄₅₋₆₇₂ were fitted using a 1:1 Langmuir binding model (not shown), and after subtraction from data obtained simultaneously from another channel of the same sensor chip, where no ligand had been immobilized. The data from EGFR₁₁₅₆₋₁₁₇₂ and EGFR_{645-672MUT} could not be fitted. At concentrations exceeding 1,000 nM, the EGFR_{645-672MUT} associated with the chip nonspecifically (not shown).

Fig. 2.

Mutations of basic residues most proximal to the inner membrane leaflet in the JD of EGFR are most critical in suppressing its phosphorylation. **a** The R→N mutations used in **b**, **c**, and **d**. **b** Western blot analysis of the membrane fraction of EGFR-expressing (EGFR) and control non-injected oocytes (NI) with anti EGFR antibodies. **c** Western Blot analysis of the membrane fraction of EGF-treated (+) and control (−) oocytes, expressing wild-type or mutant full-length EGFR, with anti phospho-EGFR (EGFR_{Y992}) and anti total EGFR antibodies. **d** Western Blot analysis of membrane extracts from EGF-treated and control oocytes, using anti phosphotyrosine (4 G10) and anti total EGFR antibodies. **e** Summary of the data on EGF-induced phosphorylation from six to eight independent experiments. Phospho-EGFR(Y992)/total EGFR ratios are expressed as a percentage of the respective ratio in the EGF-treated wild-type EGFR sample. * $P \leq 0.05$, ** $P \leq 0.005$

Fig. 3.

Mutations in the JD of EGFR impair EGF signaling to ion channels. Full length, wild-type or mutant EGFR (mutations ASN8, ASN5, and ASN3, see Fig. 2a) was co-expressed with Kir3.4-S143T (or Kir3.4*). **a** Sample traces for I_{CaCl} (upper panel) or Kir3.4* currents (lower panel) were taken from the same experiment. The membrane potential was held at -80 mV. **b** Bar graph shows Kir3.4* current levels before EGF-induced inhibition through wild-type and mutant EGFR coexpressed with the channel. Data from three experiments with three to six oocytes for each group ($N=3$, $n=3-6$) were normalized to basal Kir3.4* currents in the presence of wild-type EGFR. ($P=0.001$). **c** Bar graph combines results on Kir3.4* current inhibition by EGF from three experiments with three to six oocytes for each group ($N=3$, $n=3-6$). * $P \leq 10^{-8}$

Fig. 4.

PIP₂ levels regulate phosphorylation of heterologously expressed EGFR. **a, b** Effect of Wortmannin treatment on EGF-induced EGFR_{Y992} phosphorylation in *Xenopus* oocytes. Oocytes expressing EGFR were pretreated with DMSO vehicle (0 nM), or Wortmannin (100 nM or 15 μM) for 1 h and stimulated with EGF. **a** Membrane extracts were analyzed on WBs for phosphorylated (EGFR_{Y992}) and total EGFR. **b** Summary of the results for phosphorylated EGFR, normalized to total EGFR and expressed as % of phosphorylation of control (eight experiments). The means were significantly different (ANOVA, $p < 0.01$). **c, d** Co-expression of synaptojanin suppresses EGFR phosphorylation. **c** Western Blot analysis of membrane extracts from EGF-treated oocytes expressing EGFR±synaptojanin. **d** Mean EGF-induced EGFR_{Y992} phosphorylation was calculated from three experiments and expressed as %±SE of phosphorylation of control (t test, $p < 0.01$). **e, f** Co-expression of PIPK1α potentiates EGF-induced phosphorylation of wild-type, but not ASN8 mutant EGFR. **e** Western blot analysis of membrane extracts from EGF-treated oocytes expressing wild-type or ASN8 mutant EGFR±PIP1α. **f** Mean EGF-induced EGFR_{Y992} phosphorylation was calculated from three experiments and expressed as %±SE of phosphorylation of control (t test, $p < 0.05$).

Fig. 5.

PIP₂ levels regulate phosphorylation of native EGFR in mammalian cells. **a, b** EGFR_{Y992} phosphorylation in HeLa cells after overnight treatment with vehicle DMSO or 100 nM or 15 μM Wortmannin in serum-free medium and subsequent stimulation with EGF (100 ng/ml, 10 min). **a** Western blot analysis of the respective lysates with anti phospho- and anti total EGFR antibodies (EGFR_{Y992} and EGFR, respectively). **b** Data for EGFR_{Y992} phosphorylation (normalized for total EGFR) from four experiments were expressed as % of phosphorylation of control (*t* test, *p* < 0.05). **c** Western blot analysis of lysates from HeLa cells transfected (48–72 h) with control (nuclease-free water) or a siRNA targeting PIP5KIα and stimulated with EGF. *Bottom two panels* represent WBs for PIP5KIα and β-tubulin. **d** Bars quantify the siRNA-induced reduction in PIP5KIα. **e, f** Bars representing the effect on basal (**e**) and EGF-induced EGFR_{Y992} phosphorylation (**f**), expressed as % ± SE of control. **p* < 0.02