Phosphatidic Acid Induces Ligand-independent Epidermal Growth Factor Receptor Endocytic Traffic through PDE4 Activation

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INTRODUCTION

Endocytosis modulates EGFR function by compartmentalizing and attenuating or enhancing its ligand-induced signaling. Here we show that it can also control the cell surface versus intracellular distribution of empty/inactive EGFR. Our previous observation that PKA inhibitors induce EGFR internalization prompted us to test phosphatidic acid (PA) generated by phospholipase D (PLD) as an endogenous down-regulator of PKA activity, which activates rolipram-sensitive type 4 phosphodiesterases (PDE4) that degrade cAMP. We found that inhibition of PA hydrolysis by propranolol, in the absence of ligand, provokes internalization of inactive (neither tyrosine-phosphorylated nor ubiquitinated) EGFR, accompanied by a transient increase in PA levels and PDE4s activity. This EGFR internalization is mimicked by PA micelles and is strongly counteracted by PLD2 silencing, rolipram or forskolin treatment, and PKA overexpression. Accelerated EGFR endocytosis seems to be mediated by clathrin-dependent and -independent pathways, leading to receptor accumulation in juxtanuclear recycling endosomes, also due to a decreased recycling. Internalized EGFR can remain intracellular without degradation for several hours or return rapidly to the cell surface upon discontinuation of the stimulus. This novel regulatory mechanism of EGFR, also novel function of signaling PA, can transmodulate receptor accessibility in response to heterologous stimuli.

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Abbreviations used: EGF, epidermal growth factor; EGFR, EGF receptor; EEA1, early endosome antigen 1; CHC, clathrin heavy chain; DAG, diacylglycerol; d1Tf, diferric Tf; FIT, F-5-fluoro-2-indolyl-des-chlorohalopemide; 1-OH, 1-butanol; PA, phosphatidic acid; PAP, phosphatidic acid phosphohydrolase; PDE4, type 4 phosphodiesterases; PLD, phospholipase D; siRNA, small interference RNA; Tf, transferrin; TfR, Tf receptor; accessibility to extracellular stimulus, in response to heterologous signaling, as suggested by several studies (Salazar and Gonzalez, 2002; Vergara-Jaregui et al., 2006; Winograd-Katz and Levitzki, 2006; Zwang and Yarden, 2006). Here we studied this possibility by testing the function of phosphatidic acid (PA), which plays several roles in membrane trafficking and cell signaling (Roth and Sternweis, 1997; Wang et al., 2006).

Ligand-binding induces conformational and structural changes in the EGFR conducive to its signaling and rapid endocytosis, both depending on activation of its intracellular tyrosine-kinase domain. Ligand-bound EGFR becomes dimerized, tyrosine-phosphorylated and -ubiquitinated (Yarden and Sliwkowski, 2001), exposes endocytic motifs and recruits proteins such as AP2, Cbl, and Grb2 involved in clathrin-mediated endocytosis (Lamaze and Schmid, 1995; Wilde et al., 1999; Wiley, 2003; Sorkin and Goh, 2009). Ubiquitination very likely contributes by engaging endocytic elements with ubiquitin-interacting motifs (Eps15 and Epsin1; de Melker et al., 2004; Stang et al., 2004; Fallon et al., 2006; Kazazic et al., 2008), although it is seemingly dispensable for clathrin-mediated endocytosis (Huang et al., 2007). Ubiquitination can also promote endocytosis through yet unknown clathrin-independent pathways (Sigismund et al., 2005, 2008) and is crucial for sorting activated EGFR into the lysosomal degradation route (Madhus and Stang, 2009; Sorkin and Goh, 2009). Depending on the kind of ligand (e.g., EGF or TGF-α), and their concentration, the endocytic route taken by activated EGFR varies according to receptor ubiquitination levels (Sigismund et al., 2005, 2008;
Regulated Endocytosis of Empty EGFR

Reagents and Antibodies

We used human recombinant EGF from Invitrogen (Carlsbad, CA), Protein A-Sepharose, propanolol, and DMEEM containing high glucose from Sigma-Aldrich (St. Louis, MO), fetal bovine serum from Hyclone Laboratories (Logan, UT), forskolin from Calbiochem (San Diego, CA), enhanced chemiluminescence (ECL) system from Amersham Biosciences (Piscataway, NJ), biotin and EZLink sulfo-NHS-SS-biotin from Pierce Chemical (Rockford, IL), cell culture reagents from Invitrogen and Sigma-Aldrich, and tissue culture plastics from Nalgene Nunc (Naperville, IL). Polyclonal antibody EGF-R84 against EGF cytosolic residues 984–996 has been previously characterized (Fanuendez et al., 1992). Hybridomas producing monoclonal anti-EGF antibodies HB8506 against the EGF extracellular domain were purchased from American Type Culture Collection (ATCC; Manassas, VA). Anti-phosphotyrosine mAb 4G10 was kindly provided by Dr. Maria Rosa Bono (Universidad de Chile, Santiago, Chile). Anti-ubiquitin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Human transferrin-Alexa594 (TF-Alexa594) and lysotracker were from Molecular Probes (Eugene, OR). Monoclonal antibodies against clathrin heavy chain were from BD Transduction Laboratories (cat. no. 61000; Lexington, KY). Noncommercial reagents include dipher trans- fermin (dTF), prepared from apo-TF as described (Bali and Harris, 1996) provided by Dr. Tulo Núñez, Facultad de Ciencias, Universidad de Chile), vector pcDNA3-CAT-YFP encoding the catalytic subunit of human PKA (provided by Dr. Manuela Zaccolo, Glasgow University, Scotland, United Kingdom), PLD inhibitor 5-fluoro-2-indolyl-des-chlorohalopemide (TIPPI, Su et al., 2009; provided by Dr. Michael Frohman, Stony Brook University, NY).

Cell culture

Cells previously characterized for EGF expression and its internalization by PKA inhibitors (Salazar and Gonzalez, 2002; Buvinic et al., 2007) include permanently transfected NIH-3T3 cells expressing either wild-type EGF (Her14 cells) or a point mutant kinase-negative EGF (K721A cells; provided by Dr. Joseph Schlessinger (Yale University, New Haven, CT); Felder et al., 1990), an in-house population of HeLa cells expressing endogenous EGF, and N2a cells permanently transfected with EGF or with FLAG-tagged µ-opioid receptors (Salazar and Gonzalez, 2002). We also purchased HeLa cells from ATCC. Before the experiments, the cells were cultured to ~80% confluence and serum-starved for 24 h in media supplemented with 0.3% fetal bovine serum (FBS), unless otherwise indicated.

Indirect Immunofluorescence and Colocalization Analysis

Cells grown on glass coverslips were washed with phosphate-buffered saline (PBS) and fixed for 30 min at room temperature with 4% paraformaldehyde in PBS supplemented with 0.1 mM CaCl2 and 1 mM MgCl2, (PBS-CM). After washing three times with PBS plus 0.2% gelatin (300 Bovin; Sigma-Aldrich, PBS-CM-G) for 5 min each, the cells were permeabilized with 0.2% Triton X-100 for 10 min at room temperature and incubated for 1 h at room temperature with anti-EGF mAb 4G10 (1/1000 in PBS-CM-G), washed at least six times, and incubated in the same buffer with the secondary antibody anti-mouse IgG Alexa488 (1/1000) for 1 h at room temperature. Distribution of internalized EGF within endocytic compartments was analyzed by colocalization with Early Endosome Antigen 1 (EEA1) using a previously characterized human autoantibody (Donoso et al., 2008), human TF-Alexa594 and lysotracker, both from Molecular Probes. The cells were previously washed with ice-cold PBS and then incubated 2 h in DMEEM-HEPES serum-free media at 37°C to accumulate EGF on the cell surface. Serum-deprived cells were then preincubated with 50 µg/ml anti-EGF mAb (clone HB8506) for 30 min at 37°C and then with 10 ng/ml EGF or 100 µM propranolol for the indicated time periods. Digital fluorescence images were acquired on a Zeiss Axiovert microscope with a Plan-APOCHROMAT 63x/1.4 oil immersion objective and the 14-bit Zeiss Axiscam camera, transferred to a computer workstation running Axiovision imaging software (Zeiss, Thornwood, NY), processed, and analyzed with MetaMorph software (Universal Imaging, West Chester, PA) as described (Cancino et al., 2007; Donoso et al., 2008). All images from a single experiment were acquired under identical settings avoiding signal saturation. The percentage of colocalization, measured as integrated pixel intensity in the regions of EGF overlap with EEA1, TF-Alexa594 and lysotracker was calculated for each individual cell (n = 30–40 cells).

Flow Cytometry

Flow cytometry analyses were performed on a FACS Calibur flow cytometer using CellQuest software (BD Biosciences, San Jose, CA). Cell surface EGF

Roeptorstf et al., 2009). Therefore, endocytic trafficking of ligand-activated EGFR can be regulated at different levels with important signaling implications (Madshus and Stang, 2009; Roeptorstf et al., 2009).

Less known regulatory mechanisms operate upon empty/inactive EGFR. In the absence of ligand, phospholipase-mediated by PKA down-regulation. Therefore, signaling pathways emerging from a variety of receptors might hypothetically transmodulate the permanence of empty/inactive EGFR at the cell surface. Because PKA has been involved in vesicular trafficking (Wojtal et al., 2008) and mediates cross-talk with multiple signaling pathways (Houslay and Adams, 2003), it seems especially suitable for integrating signals toward endocytic transmodulation of EGFR cell surface expression. It is then interesting to identify endogenous signaling pathways able to down-regulate PKA activity and induce ligand-independent EGFR internalization mimicking the effects of PKA inhibitors (Salazar and Gonzalez, 2002).

PA, which has a little known function as upstream regulator of the cAMP/PKA pathway (Grange et al., 2000), is an interesting candidate. PA is a membrane component present at low levels, which through transient and regulated increments, can promote membrane trafficking and signal transduction (Wang et al., 2006; Roth, 2008). PA is mainly generated by phospholipase D (PLD) activity, but can also derive from phosphorylation of diacylglycerol (DAG) by DAG kinase, and is consumed by phosphatidic acid phosphohydrolases (PAP) producing DAG (Wang et al., 2006). Mammalian cells express two isoforms of PLD, PLD1 and PLD2, both involved in membrane trafficking (Roth, 2008; Donaldson, 2009) and signaling from a variety of receptors (Liscovitch et al., 2000; Wang et al., 2006), including EGF (Zhao et al., 2007). In membrane trafficking, local increases in PA levels are believed to confer special structural membrane properties and to recruit and modulate essential elements of vesicular traffic (Jones et al., 1999; Roth, 2008), in addition to serve as substrate for DAG production, which also participates in vesicle generation at least in certain membranes (Baron and Malhotra, 2002; Asp et al., 2009). Ligand-induced endocytosis of EGFR involves PLD-generated PA (Shen et al., 2006; Lee et al., 2006, 2009). In signal transduction, PLD activity is increased by a large variety of stimuli and receptor pathways (Liscovitch et al., 2000; Wang et al., 2006; Zhao et al., 2007) and the generated PA recruits and modulates signaling components bearing PA-binding domains, such as SOS and Rap (Stace and Kistakas, 2006; Wang et al., 2006; Zhao et al., 2007). One of the PA’s signaling pathways relatively ignored in the field of membrane trafficking is directly linked to the activity of the cAMP/PKA system (Grange et al., 2000). cAMP levels are finely regulated by adenylcyclases that produce cAMP and phosphodiesterases (PDEs) that degrade it (Houslay and Adams, 2003). PA binds and activates long isoforms of rolipram-sensitive type 4 PDEs (PDE4), provoking a decrease in cAMP levels and down-regulation of PKA activity (Nemoz et al., 1997; Grange et al., 2000). Therefore, we propose the hypothesis that PA constitutes an endogenous upstream modulator of empty/inactive EGFR endocytosis mediated by PKA down-regulation.

We show that PAP inhibition with propranolol, currently used to assess the function of PA and DAG in cellular processes (Baron and Malhotra, 2002; Jovanovic et al., 2006;
was assessed by incubating the cells with the monoclonal anti-EGFR antibody HB8506 (1.5 dilution of a 4-d hybridoma cell culture media) for 30 min at 4°C, washed twice with PBS, and then incubated with rabbit anti-mouse Alexa-488 antibodies (1:1000) for 30 min at 4°C. Cells were detached from the tissue culture with PBS-EDTA, and fluorescence was acquired by flow cytometry, analyzing 20,000 living cells. To assess EGFR return to the cell surface from previously endocytosed pool, the cells were first incubated with primary antibodies for 30 min at 4°C, washed twice, and then incubated for 30 min at 37°C in the presence of transferrin to induce endocytosis. After acid wash at 4°C, to move the primary antibodies that remained attached to the receptor at the cell surface, the cells were reincubated at 37°C for different time periods, and the EGFR-immune complexes that reappeared at the cell surface were chased with secondary antibodies and analyzed by flow cytometry. Data were analyzed using WinMDI 2.9 software (Windows, Microsoft, Redmond, WA).

Transfection and Silencing with Small Interference RNA
For expressing the catalytic subunit of PKA coupled to YFP (PKA-YFP), HeLa cells were transiently transfected with Lipofectamine 2000 according to the manufacturer’s protocol, using either 1 μg of plasmid for 6 or 0.3 μg for 18 h. Only cells with normal morphology were analyzed. Small interference RNAs (siRNAs) were purchased from Dahlmacon (Boulder, CO), using the commercial on-target plus smart pool L-004001-01-005 for clathrin heavy chain (CHC; Gene accession number NM_002662). For PLD2 we used the described sequence (Padron et al., 2006) aagaggguccggagguuggg (Gene accession number NM_002663). Controls siRNA were siGENOME non-targeting siRNA 2 (D-001210-02-05) and D-001210-04-05. HeLa cell were in 60–70% confluence were transfected twice at 24-h intervals, using 100–200 pmol of the corresponding siRNAs and Lipofectamine 2000 under the manufacturer’s recommendations. Because no commercial antibodies are available for distinguishing PLD1 and PLD2 expression, RT-PCR was used with the following primers: PLD1: forward; caacagcaacagctgag; reverse, AATCA-CACCCATTCCATGTC; and PLD2: forward, AAAGATATACCCGG-GATCC; reverse, CAGGTATTTCTGTTGCTGC. Expression of CHC was analyzed by immunoblot with a mAb (BD Transduction Laboratories) on 50 μg of cell extract.

RESULTS

PAP Inhibition Induces Internalization of EGFR in the Absence of Ligand
Propranolol has been reported to increase PA levels leading to PDE4 activation and PKA down-regulation (Grange et al., 2000). Therefore, we first tested whether it can reproduce the effects of PKA inhibitors on EGFR distribution. HeLa cells treated with different concentrations of propranolol for 30 min showed decreased 125I-EGF binding activity, up to ~80% at 250 μM, with half maximal (50% increase) concentration of ~75–100 μM, depending on the propranolol batch (Figure 1A, left). It is very likely that propranolol at 250 μM almost completely inhibited the PAP activity (Asp et al., 2009). Higher concentrations provoked cell detachment. We then used concentrations of 75–100 μM for the rest of the experiments. Time-course experiments with 100 μM propranolol showed that 125I-EGF binding decreases with t1/2 ~13–15 min, measured within the first 10 min to avoid recycling (Figure 1A, right and inset). After decreasing to ~60%, it increases again to a plateau of ~50% initial binding (Figure 1A, right). It seems that empty EGFR is first rapidly removed from the cell surface by an accelerated endocytosis, and then the balance of its internalization and recycling establishes a new steady state. Because empty EGFR has been shown to recycle very fast, predominantly with t1/2 ~5 min (Wiley, 2003), it is likely that the new steady state results from both an accelerated endocytosis and a decreased recycling. Scatchard analysis showed that our in-house preparation of HeLa cell expresses ~300,000 receptors per cell and both high- and low-affinity binding sites decreased under propranolol treatment, as previously described for N2a cells treated with PKA inhibitors (Salazar and Gonzalez, 2002). Similar results were obtained in HeLa cells purchased from ATCC, which express ~40,000 receptors per cell (not shown). The decreased binding was due to EGFR internalization. Indirect immunofluorescence showed redistribution of EGFR from the cell surface to juxtanuclear endosomal compartments, mimicking the effect of the specific PKA inhibitor PKI-Myr (Figure 1B). At this time point, the slightly different immunofluorescence pattern seen for the PKA inhibitor is due to its fastest effect. All these results are congruent with the possibility that the described PA/PKA link (Grange et al., 2000)
(2000) is involved in endocytic control of EGFR cell surface expression.

Propranolol Induces Internalization of Inactive EGFR Independent of Tyrosine Phosphorylation and Ubiquitination

Because EGFR is transactivated by a variety of stimuli (Carpenter, 1999) and its endocytosis has been shown to involve tyrosine phosphorylation and ubiquitination (Wiley, 2003; Sigismund et al., 2005; Wang et al., 2005; Sorkin and Goh, 2009), we studied whether these modifications occur under propranolol treatment. Neither tyrosine phosphorylation nor ubiquitination could be detected (Figure 2A). Furthermore, propranolol induced internalization of the kinase-death K721A receptor, which is not only inactive but also not ubiquitinated (Felder et al., 1990; Figure 2B). Therefore, similar to PKA inhibitors (Salazar and Gonzalez, 2002), EGFR internalization triggered by propranolol does not require receptor tyrosine phosphorylation or ubiquitination.

PLD-generated PA Induces Internalization of EGFR

Next, we studied whether propranolol-induced EGFR internalization can be attributed to PLD-generated PA. Propranolol (75–100 μM) caused a transient increase of PA levels (Figure 3A), which became threefold higher in 20 min and then decreased to almost basal levels. Sensitivity to primary alcohols is currently used to test the involvement of PLD-generated PA in cellular processes (Kahn et al., 1993; Ktistakis et al., 1995; Jones et al., 1999). PLD preferentially uses primary but not secondary alcohols over water in a transphosphatidylation reaction, producing phosphatidylbutanol instead of PA (Moritz et al., 1992). Pretreating HeLa cells with 1% 1-butanol almost completely reduced the increment of PA (Figure 3A), as well as EGFR internalization (Figure 3C), whereas 2-butanol had no effect (not shown). FIPI, an inhibitor of both PLD1 and PLD2 (Su et al., 2009) also counteracted the EGFR internalization (Figure 3C). However, this approach has the limitation that a strong inhibition of PA generation can restrict the AP2-mediated recruitment of clathrin to the plasma membrane and thus inhibit this endocytic pathway (Boucrot et al., 2006). 1-Butanol at higher concentrations (1.5–2%) can lower the activity of phosphatidylinositol 4-phosphate 5-kinase (PIP5K) normally stimulated by PA, decreasing phosphatidylinositol-4,5-bisphosphate (PIP2) production and the subsequent recruitment of AP2 and clathrin to the plasma membrane.

Figure 1. PAP inhibition by propranolol induces internalization of EGFR, mimicking the effect of PKA inhibitors. (A) Dose-dependent effect of propranolol on 125I-EGF binding (left). Right panel shows time course with 100 μM propranolol, and the inset shows in detail the kinetics of decrease for the first 10 min (average ± Standard Error of the Mean (SEM) of five independent experiments in triplicate). (B) Indirect immunofluorescence of EGFR. HeLa cells incubated in the absence (Control) or presence of EGF (50 ng/ml) for 10 min, PKA inhibitor PKA-Myr peptide (20 μM), or propranolol (75 μM) for 30 min at 37°C were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and analyzed with HB8506 anti-EGFR antibody. Bar, 10 μm.
the presence of either 50 ng/ml EGF (lanes 2–5) or 75nation of EGFR. HeLa cells were incubated in the absence (lane 1) or and is internalized independently of its tyrosine-kinase activity. (A) Figure 2. Under propranolol treatment, EGFR is not transactivated and is internalized independently of its tyrosine-kinase activity. (A) Propranolol does not induce tyrosine phosphorylation or ubiquitination of EGFR. HeLa cells were incubated in the absence (lane 1) or presence of either 50 ng/ml EGF (lanes 2–5) or 75 μM propranolol (lanes 6–9) for the indicated time periods at 37°C. EGFR was then immunoprecipitated with mAb-HB8506, and its phosphotyrosine (top) and ubiquitin (bottom) content were assessed by immunoblot with mAb 4G10 anti-phosphotyrosine or anti-ubiquitin mAb P4D1. Bottom, the total mass of EGFR detected after stripping and incubating the blots with polyclonal antibody EGFR984. In contrast with EGF, propranolol does not induce tyrosine phosphorylation or ubiquitination of EGFR. (B) Propranolol induces internalization of K721A kinase-minus EGFR. Indirect immunofluorescence of Her 14 cells expressing either wild type or K721A kinase-minus EGFR shows similar intracellular distribution of both receptors after 30 min of propranolol (75 μM) treatment.

(Boucrot et al., 2006). In our HeLa cells treated with 1% 1-butanol, we did not detect a significant change in clathrin distribution by confocal microscopy analysis of the plasma membrane surface attached to substrate (not shown). However, to avoid a blocking effect on clathrin-mediated endocytosis, which would shield an effect of propranolol mediated by any signaling pathway leading to EGFR endocytosis, we tested two other assays. PLD2 has been described to cytosis, which would shield an effect of propranolol mediated by any signaling pathway leading to EGFR endocytosis, we tested two other assays. PLD2 has been described to account for most of the constitutively generated PA and its silencing by siRNA inhibits recycling but not endocytosis of TfR (Padron et al., 2006), indicating an unaffected clathrin-mediated endocytosis. In our conditions, transfection with siRNA specifically decreased mRNA expression levels of either PLD1 (~76%) or PLD2 (~64%) assessed by RT-PCR (Figure 3B). Silencing PLD2, but not PLD1, counteracted ~50–60% of the propranolol effect on [125I]-EGF binding (Figure 3C). On the other hand, to obtain more direct evidence that PA itself promotes EGFR internalization we added PA to cells in the form of micelles (400 μg/ml) for 30 min at 37°C. This treatment decreased [125I]-EGF binding activity in HeLa cells (Figure 3D). Similar results were obtained in Her14 cells, which are NIH3T3 cells permanently transfected to express wild-type EGFR (Felder et al., 1999). About 50% of the Her14 cells incubated with PA-micelles show immunofluorescence patterns characteristic of internalized EGFR (Figure 3D). Therefore, increases in PA achieved either by PAP inhibition or PA-micelles provoke internalization of empty EGFR.

**EGFR Internalization Induced by PA Involves a PDE4/cAMP/PKA Pathway**

Increasing the PA levels with propranolol has been shown to decrease cAMP levels and PKA activity due to activation of PDE4 (Grange et al., 2000). In HeLa cells, we corroborated that propranolol increases the PDE activity and found this increment completely abrogated by the PDE4 inhibitor rolipram (Figure 4A). Propranolol also decreased the cAMP levels and PKA activity (Figure 4, B and C). The magnitude and timing of the detected changes seems not entirely related. For instance, the activity of PDE reached a peak within 10 min and then decreased to slightly above the basal levels. PKA activity decreased 70% within 10 min, in congruency with the changes in PDE activity, but then remained low during the rest of the experiment. The levels of cAMP showed a more gradual decrease, reaching ~70% in 30 min. Indeed, our assays detect only global changes that certainly do not reflect the operation of a highly compartmentalized and interregulated PDE4/cAMP/PKA system (Willoughby and Cooper, 2008). Several evidences indicated that this PDE4/cAMP/PKA pathway is the main mediator of PA induced EGFR internalization. The specific inhibition of PDE4 isoforms by rolipram counteracted ~75–80% of the propranolol-induced EGFR internalization. The usual decrease of ~50% in the [125I]-EGF–binding activity elicited by propranolol was now reduced to only ~10–15% in the presence of 30 μM rolipram, leaving unaffected the EGF-induced internalization and thus discarding deleterious effects (Figure 4D). The counteracting action of rolipram was also observed by immunofluorescence (Figure 4E). To discard possible off-target effects, we additionally interfered with the PDE4/cAMP/PKA system at two other levels: activating adenylyl cyclases by forskolin and overexpressing the catalytic subunit of PKA. Forskolin inhibited ~70% of the EGFR internalization induced by propranolol (Figure 4F). The experiments of PKA overexpression were performed adjusting the conditions to avoid expected toxic effects. Transfections with either 1 μg of PKA-YFP vector for 6 h or 0.3 μg of vector for 18 h showed 5–10% of the cells displaying clearly detectable yellow fluorescent protein (YFP) fluorescence without signs of cell damage. Under these conditions, we consistently observed that the characteristic immunofluorescence pattern of EGFR endocytosis appeared very infrequently (<3%) in cells expressing PKA-YFP, whereas most (>90%) adjacent cells, not expressing PKA-YFP, showed this pattern (Figure 4G). All these results strongly indicate that PKA signaling toward PKA down-regulation, via activation of rolipram-sensitive PDE4s, constitutes the main pathway leading to EGFR internalization.

**The Endocytic Effect of Propranolol Is Selective**

The effect of PKA inhibitors that induce EGFR internalization is selective (Salazar and Gonzalez, 2002); e.g., neither affect the
internalization rate of Tf, which is constitutively endocytosed via the clathrin pathway (Maxfield and McGraw, 2004), nor the distribution of the \( /H9262\)-opioid receptor, which is also endocytosed by the clathrin pathway but in response to agonist (DAMGO) stimulation (Keith et al., 1996). IN/SUR analysis (Wiley and Cunningham, 1982) of \(125\text{I}-\text{Tf}\) endocytosis showed an endocytic constant (\(k_e\)) of 0.12 ± 0.01 min\(^{-1}\) that did not significantly change in propranolol-treated cells (\(k_e = 0.19 ± 0.034\) min\(^{-1}\); Figure 5A). However, when cells were incubated with Tf-Alexa594 for longer times, 30 – 60 min, fluorescence was seen accumulated in juxtanuclear recycling compartments, suggesting that propranolol decreases the recycling phase of TfR (Figure 5B).

Previously characterized N2a cells stably transfected with either the EGFR or \(\mu\)-opioid receptor (Salazar and Gonzalez, 2002), which lack caveolin-1 expression and do not form caveolae at their cell surface (Gorodinsky and Harris, 1995), showed propranolol-induced internalization of EGFR (Figure 5C; top panels) but not \(\mu\)-opioid receptor, which was otherwise internalized by stimulation with DAMGO (Figure 5C; bottom panels). Therefore, EGFR endocytosis induced by propranolol is not due to an indiscriminate enhancement of membrane internalization. It is selective and can occur independently of caveolin-1 and caveolae.

**Activation of the PA/PKA Pathway Increases Endocytosis (Clathrin-dependent and -independent) and Decreases Postendocytic Recycling of Empty EGFR**

Cell surface distribution of empty EGFR has been shown to result from internalization at low rates (\(t_{1/2} \sim 20–30\) min) and a predominant fast component (\(t_{1/2} \sim 5\) min) of recycling (Herbst et al., 1994; Wiley, 2003). Thus, accelerated endocytosis, decreased recycling, or both can lead to EGFR internalization. Accelerated endocytosis by propranolol treatment was already

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**Figure 3.** PA generated by PLD2 mediates EGFR internalization induced by propranolol. (A) PAP inhibition by propranolol provokes a transient increase of PA levels. HeLa cells were pulse-labeled with \[^{3}H\]myristic acid (10 \(\mu\)Ci) for 16 h and then treated with 75 \(\mu\)M propranolol for 30 min at 37°C. PA levels were resolved by TLC (top) and quantified by scan densitometry. The inhibitory effect of coincubation with 1-butanol (1-OH) is shown for the 20-min time point (top, last lane; bottom, triangle in the graph). (B) RT-PCR for PLD1 and PLD2 mRNAs in HeLa cells transfected twice with the corresponding siRNA for PLD1 or PLD2. The bands were quantified by densitometry (\(p < 0.001\); two-tailed Student’s t test). (C) Countering effects of PLD inhibition or silencing. The decrease in \(125\text{I}-\text{EGF}\) binding induced by 100 \(\mu\)M propranolol (100% effect) is counteracted ~50% by FIP1, 1-butanol and transfection with siRNA for PLD2 but not PLD1 (\(p < 0.001\); **p < 0.01). (D) PA directly added in micelles induces EGFR internalization. HeLa cells incubated with PA micelles (400 \(\mu\)g/ml) for 30 min at 37°C show a ~30% decrease in the levels of \(125\text{I}-\text{EGF}\) binding (average ± SEM; \(p < 0.001\)). (E) Indirect immunofluorescence of Her14 cells incubated with PA micelles (400 \(\mu\)g/ml) for 30 min at 37°C shows EGFR redistribution from the cell surface to intracellular compartments. Bar, 10 \(\mu\m).
suggested by the rate of ligand-binding decrease ($t_{1/2} \approx 13–15$ min; see Figure 1A, inset). The contribution of an eventually inhibited recycling should be minimal within the 10-min period used to estimate the $t_{1/2}$. We obtained further evidence of enhanced endocytosis with a biotinylation assay using cleavable sulfo-NHS-SS-biotin (Salazar and Gonzalez, 2002; Burgos et al., 2004). Cell surface proteins were first labeled with sulfo-NHS-SS-biotin at 4°C. Then, after shifting the temperature to 37°C, the pool of biotinylated EGFR remaining at the cell surface can be distinguished from that internalized by sensitivity to reduction with glutathione (Figure 6A). Cells treated just 5 min with propranolol showed three- to fourfold higher mass of internalized EGFR than control cells (Figure 6B). Therefore, propranolol clearly increases the endocytic rate of empty/inactive EGFR.

Activated EGFR can be endocytosed via clathrin-dependent and -independent pathways, depending on its stimulation with either low or high ligand concentrations, which in turn provokes receptor tyrosine phosphorylation without or with detectable ubiquitination, respectively (Sigismund et al., 2008). To assess the role of the clathrin-mediated pathway we performed knockdown experiments with siRNA for CHC (Huang et al., 2004; Sigismund et al., 2008). A two-round transfection protocol, with 24-h intervals, decreased >95% clathrin expression, as assessed by immunoblot 48 h after the first transfection (Figure 6C). The CHC-silenced
cells showed increased (40%) levels of EGF binding, likely reflecting a clathrin-mediated endocytic deficiency. However, these cells showed only ~50% reduction of the propranolol effect. In these experiments, propranolol (100 μM) treatment decreased the 125I-EGF-binding activity by ~60–70% in the cells transfected with control siRNA and ~30% in the cells transfected with CHC siRNA (Figure 6C, graph). Thus, accelerated EGFR endocytosis induced by propranolol in the absence of ligand seems to involve both clathrin-dependent and -independent pathways.

The experiment depicted in Figure 5B, showing accumulation of Tf in juxtanuclear compartments, suggested a non-selective inhibitory effect of propranolol on recycling traffic. To further study this possibility, we performed two assays that measured the cell surface return of EGFR previously internalized under propranolol treatment. EGFR was first labeled at the cell surface with either cleavable sulfo-NHS-SS-biotin or mAb HB8506, which interacts with the receptor extracellular domain. Then, the cells were treated for 30 min with propranolol to induce receptor endocytosis, and the label remaining at the cell surface was stripped by glutathione reduction or acid wash at 4°C. After this treatment, the cells were incubated for 30 min more in the presence or absence of propranolol. In the biotinylation assay, EGFR remaining intracellular was assessed by resistance to glutathione and was found to be three to four times higher in the cells maintained under propranolol treatment after the initial internalization step (Figure 6D). Flow cytometry analysis of cells incubated with HB8506 antibody gave a more quantitative picture. Progressive cell surface reappearance of HB8506–EGFR complex occurred within the first 10–15 min, reaching a plateau at ~20% regardless of propranolol treatment (Figure 6E). During this time period, the PA/PKA pathway presumably remains operative even in the absence of drug. Afterward, a clear difference became apparent. In the absence of propranolol most of the previously internalized antibody-EGFR complex reappeared at the cell surface, whereas in the presence of propranolol almost 80% remained intracellular (Figure 6E). Considering all, these results indicate that propranolol treatment decreases the postendocytic recycling of EGFR.

In summary, internalization of empty EGFR induced by propranolol results from both increased endocytosis and decreased recycling. The endocytic phase involves clathrin-dependent and -independent pathways. Interestingly, propranolol-induced internalization is almost completely reversible upon discontinuing the stimulus of the PA/PKA pathway. Thus, this PA/PKA pathway provides a fast and transient mechanism for controlling EGFR expression levels at the cell surface.

**Figure 5.** Propranolol does not affect endocytosis of TIR and μ-opioid receptors, but promotes intracellular accumulation of Tf-Alexa 549. (A) Constitutive endocytosis of TIR. HeLa cells were preincubated in serum-free media for 1 h at 37°C and then with 20 ng/ml 125I-diTf for 2 h at 4°C. After eliminating the unbound 125I-diTf, cells were incubated in the presence or absence of 75 μM propranolol for 5–10 min at 37°C. IN/SUR plot shows no difference in the internalization rates (ke). (B) Propranolol increases the accumulation of Tf-Alexa 549 in juxtanuclear endosomes. Cells incubated with 50 μg/ml Tf-Alexa 549 at 37°C for 30 min in the absence or presence of 75 μM and fixed for fluorescence imaging show strong juxtanuclear fluorescence accumulation, suggesting decreased TIR recycling. (C) Propranolol treatment does not cause redistribution of μ-opioid receptors. N2a cells permanently transfected with either EGFR or FLAG-tagged μ-opioid receptors were incubated with either 75 μM propranolol or 10 μM DAMGO (μ-opioid receptor ligand), for 30 min. Bar, 10 μm.
HeLa cells were first incubated for 1 h with either 50 μg/ml Tf-Alexa 594, to load sorting and recycling endosomes, or with 1 μM lysotracker, to label late endosome/lysosomal compartments. In response to EGF, the EGFR entered first into EEA1- and Tf-Alexa 594–containing endosomes, reaching 60% colocalization with these markers in 5 min. Afterward, EGFR gradually left this compartment and started to colocalize with lysotracker, indicating segregation into the lysosomal degradation pathway (Figure 7B). In contrast, under the effect of propranolol, empty EGFR became detectable in EEA1- and Tf– containing sorting endosomes within 15 min, reached a peak in 60 min, and then left the EEA1 compartment and started to colocalize with Tf-Alexa 594, progressively accumulating in juxtanuclear compartments that correspond to recycling endosomes (Figure 7C). Interestingly, these Tf-Alexa 594–containing juxtanuclear endosomes were frequently seen enlarged, suggesting that recycling of TiR, as well as empty EGFR, might also be decreased, as already suggested (see Figure 5B).

These results indicate that the PA/PKA pathway triggered by propranolol drives empty EGFR first into sorting endosomes, similarly to ligand-activated EGFR, but then the receptor diverges from the lysosomal pathway, as expected for a nonubiquitinated receptor, and accumulates in juxtanuclear recycling endosomes escaping degradation for several hours.

**DISCUSSION**

In this work we provide evidence that cell surface versus intracellular distribution of empty/inactive EGFR can be regulated by PA signaling independently of ligand. Acute increase in PA levels induces internalization of EGFR...
Figure 7. EGFR internalized during propranolol treatment accumulates in recycling endosomes without degradation. (A) EGFR mass under EGF or propranolol treatment. Immunoblots with polyclonal antibody EGFR984 and densitometric analysis (graph) show an almost unchanged EGFR mass for up to 4 h of propranolol (75 μM) treatment, instead of the progressive decrease (t1/2 ~60 min) seen during incubation with EGF (50 ng/ml). (B and C) Immunofluorescence colocalization analysis of internalized EGFR. HeLa cells preincubated with 50 μg/ml Tf-Alexa 549 or 1 mM lysotracker at 37°C for 1 h were then treated with either 10 ng/ml EGF (B) or 100 μM propranolol (C) for the indicated time points and then processed for indirect immunofluorescence with anti-EGFR mAb HB8506 (green) and anti-EEA1 polyclonal antibody. Merged digitalized images of representative patterns are shown. Graphs represent the quantitative analysis of EGFR colocalization with each marker. Bar, 10 μm.
through PDE4-mediated down-regulation of PKA activity. Internalized EGFR accumulates in recycling endosomes and can either stay there without degradation for several hours or return to the cell surface when PA signaling is discontinued. This constitutes a novel control mechanism of EGFR function. Transmodulation of EGFR distribution that changes its accessibility to external stimulus can occur through this PA/PKA pathway. This is also a novel function for the described link between signaling PA and PKA (Grange et al., 2000).

Our previous observation that inhibition of basal PKA activity induces internalization of inactive EGFR (Salazar and Gonzalez, 2002) led us to explore whether PA signaling toward activation of rolipram-sensitive PDE4s (Grange et al., 2000) constitutes an endogenous upstream element of this pathway. We used the current approach of inhibiting PAP activity with propranolol to produce an increase in PA levels (Grange et al., 2000; Baron and Malhotra, 2002; Asp et al., 2009). Propranolol induced a concentration-dependent internalization of EGFR, up to ~80% at the highest concentration, associated with a transient increase in PA levels. A combination of experiments that interfered with PA production by PLD, using inhibitors and specific silencing of PLD2 mRNA with siRNA, which in contrast with 1-butanol (Boucrot et al., 2006) does not affect clathrin-mediated endocytosis (Padron et al., 2006), together with direct addition of PA micelles, allow to conclude that an increment in PA levels is able to induce internalization of EGFR in the absence of ligand and mediates the effect of propranolol.

To explore the process by which EGFR becomes internalized in more detail, we analyzed the endocytic and recycling phases separately. Empty EGFR is constantly internalized at low rates ($t_{1/2}$ ~20–30 min) and recycles rapidly, with a predominant fast component of ~5 min and a more prolonged phase of ~20 min (Wiley, 2003). Intracellular EGFR accumulation can thus occur through accelerated endocytosis, decreased recycling, or both. Under propranolol treatment, ligand-binding activity decayed with a lower $t_{1/2}$ ~13–15 min. Binding assays probably underestimate the endocytic rate due to the cell surface returning of the receptors already present in intracellular compartments. Biotinylation assays using cleavable sulfo-NHS-SS-biotin consistently showed three- to fourfold more mass of EGFR internalized within 5 min of propranolol treatment than in control conditions. Thus, propranolol clearly accelerates endocytosis of empty EGFR. To assess the recycling phase, we labeled the EGFR at the cell surface with cleavable sulfo-NHS-SS-biotin or with a mAb before inducing its internalization by propranolol treatment. By sensitivity to glutathione or by flow cytometry analysis, we assessed whether the internalized EGFR returns to the cell surface in the absence or presence of propranolol. Almost a complete return was observed when propranolol was removed from the medium; otherwise EGFR remained intracellular, reflecting an inhibition of its recycling. The internalized EGFR can stay intracellular without detectable degradation for several hours. These results indicate that propranolol induces rapid and reversible changes on EGFR cell surface levels by both accelerating endocytosis and decelerating recycling. These two effects are probably mediated by different mechanisms, as the endocytic phase is selective for EGFR, whereas the recycling phase also seems to affect TIR.

How an increase in PA levels accelerates endocytosis of EGFR in the absence of ligand remains unknown. PA changes the physical properties of membranes: charge, pH, and curvature, facilitating vesicle generation and fusion, and also recruits and activates proteins directly involved in the trafficking machinery (reviewed in Jones et al., 1999; Roth, 2008). PA-mediated activation of PIP5K produces PIP2-binding sites for the clathrin adaptors AP2 and AP180/CLM (Padron et al., 2003; Honing et al., 2005; Boucrot et al., 2006), both involved in clathrin-mediated endocytosis of ligand-activated EGFR (Sorkin and Goh, 2009). PIP5K overexpression has been reported to increase the endocytic rate of TIR (Padron et al., 2003). However, we did not observe an accelerated rate of TIR endocytosis, suggesting that the increment in PA levels produced by the low propranolol concentrations used in our experiments (75–100 μM) are not enough to provoke detectable improvements of clathrin coat dynamics. On the other hand, AP2 is expected to interact only with activated EGFR upon exposition of endocytic motifs (Boll et al., 1995; Sorkin et al., 1995; Sorkin and Goh, 2009), but we found no signs of EGFR activation under propranolol treatment. Neither tyrosine phosphorylation nor ubiquitination could be detected. Propranolol also induced internalization of the K721A mutant receptor lacking tyrosine kinase activity. These results completely discard transactivation as the cause of EGFR internalization and indicate that propranolol induces internalization of empty/inactive EGFR. μ-opioid receptor, which is also endocytosed by the clathrin pathway in response to agonists, did not change its distribution under propranolol treatment. Therefore, we can discard a generalized effect derived from PA-mediated changes in membrane properties and clathrin coat dynamics. At least the endocytic phase of PA-induced internalization of empty/inactive EGFR has a selective mechanism, which should promote receptor interaction with the endocytic machinery.

Strikingly, siRNA experiments that silenced >95% of clathrin levels decreased only 50–60% of the propranolol-induced EGFR internalization. Propranolol also induced EGFR internalization in N2a cells that do not express caveolin and do not assemble caveolae at their cell surface (Gordon and Harris, 1995). Therefore, close to half of the empty/inactive EGFR becomes endocytosed via the clathrin-dependent pathway, implying an unknown mechanism of interaction with clathrin coats. The rest of the endocytosis likely occurs via some of the described clathrin-independent routes (Howes et al., 2010), which in this case would not require caveolin or caveolae. Clathrin-independent endocytosis has been involved in several cellular processes (Grant and Donaldson, 2009; Howes et al., 2010), recently including the endocytosis of EGFR stimulated by high EGF concentration that conduces to receptor degradation and signal attenuation (Sigismund et al., 2008). Our results add a new role: the removal of empty/inactive EGFR from the cell surface in response to heterologous stimuli that increase PA levels.

In contrast with the selective influence on the endocytic phase, the inhibitory effects of propranolol on recycling might be more generalized. Quantitative immunofluorescence colocalization analysis showed that EGFR internalization during propranolol treatment enters first into early sorting endosomes, similar to ligand-activated EGFR (Sorkin and von Zastrow, 2009) and to many clathrin-independent cargo (Howes et al., 2010), but then diverges from the lysosomal degradation route taken by activated EGFR and progressively accumulates in juxtanuclear recycling endosomes. From these compartments recycling can occur at lower rates than from sorting endosomes (Maxfield and McGraw, 2004; Grant and Donaldson, 2009), but still might be impaired by the PA pathway elicited by propranolol. An enlargement of TF-containing recycling endosomes suggests that inhibition of TIR recycling also occurs under propranolol treatment. Membrane recycling of cargo endocytosed via clathrin-independent pathways seems to depend on Arf6-mediated acti-
Regulated Endocytosis of Empty EGFR

vation of PLD (Donaldson, 2009; Grant and Donaldson, 2009) and therefore, should be stimulated by PA. Therefore, recycling routes followed by empty/inactive EGFR might be shared by TIR but not by another cargo. This possibility would require further analysis studying other recycling receptors and the role of PLD.

We corroborated that propranolol increases PDE activity and consequently decreases cAMP levels and PKA activity in HeLa cells, as described in other cells (Grange et al., 2000). The magnitude and timing of the PDE and PKA activities and cAMP levels seemed somehow unrelated, but our assays certainly do not reflect the subtle changes occurring in a highly compartmentalized PDE4/cAMP/PKA system (Houslay and Adams, 2003; Willoughby and Cooper, 2008). However, we found strong counteracting effects upon interfering with the PKA system at different levels, e.g., inhibition of PDE4 activity with rolipram, activation of adenyllylcyclases with forskolin, and overexpression of the catalytic subunit of PKA. These data constitute a robust evidence that PDE4-mediated down-regulation of the cAMP/PKA system is the crucial event leading to EGFR internalization. The mechanism remains unknown.

PKA has been widely involved in a variety of vesicular trafficking processes but generally through activation rather than inhibition (Wojtal et al., 2008). PKA substrates remaining to be identified might determine a predominant distribution of EGFR on the cell surface promoting its escape from the endocytic machinery until basal PKA activity is down-regulated. For instance, PKA might negatively regulate the p38 kinase activity that has been associated with EGFR internalization (Vergara-Jauregui et al., 2006; Winograd-Katz and Levitzki, 2006; Zwang and Yarden, 2006). Interestingly, PKA can phosphorylate serines in the cytosolic domain of EGFR (Barbier et al., 1999) and can also directly interact with the ligand-activated EGFR through Grb2 protein (Tortora et al., 1997), which is involved in ligand-activated EGFR endocytosis (Wang and Moran, 1996; Huang and Sorkin, 2005). The role of these events and perhaps other element(s) acting downstream of PKA in empty/inactive EGFR endocytosis and recycling are the challenges for future studies.

Finally, our results expand the described roles of the PA/PKA pathway as well as the role of PLD in EGFR endocytosis. The functional relevance of the PA/PKA pathway so far has been restricted to T lymphoid cells in which cAMP is a major negative regulator (Marcoz et al., 1993; El Bawab et al., 1997; Zakaroff-Girard et al., 1999; Norambuena et al., 2009). Endocytic trafficking involving PLD-generated PA include ligand-induced endocytosis of EGFR (Shen et al., 2001; Lee et al., 2006, 2009), angiotensin-II (Du et al., 2004), and μ-opioid (Koch et al., 2004), constitutive endocytosis of the metabolotropic glutamate receptor (Bhattacharya et al., 2004), constitutive recycling but not endocytosis of TIR (Padron et al., 2006), and ARF6-mediated membrane recycling from a clathrin-independent endocytic pathway (Grant and Donaldson, 2009). The results shown here suggest that PA can potentially transmodulate the EGFR not through the classical transactivation mechanisms (Carpenter, 1999), but through endocytic removal from the cell surface in response to a variety of stimuli that activate PLD (e.g., various hormones, growth factors, cytokines, neurotransmitters, adhesion molecules, drugs, and physical stimuli; Liscovitch et al., 2000). On the other hand, it has been proposed that low concentrations of EGF stimulate PLD1 to exert a GTPase-activating effect on dynamin, enhancing EGFR endocytosis without increasing the PA levels (Lee et al., 2006). At high EGF concentrations, PLD1 activation generates PA, which through an autoregulatory loop promotes interaction of PLD1 with μ2 leading to recruitment of AP2 and faster EGFR endocytosis (Lee et al., 2009). We have previously shown that high EGF concentrations down-regulate PKA activity (Salazar and Gonzalez, 2002). It is then possible that the PA/PKA pathway triggered by a strong PLD activation might contribute to the removal of EGFR from the cell surface, not only in response to heterologous stimuli but also during ligand-induced EGFR activation. This control system can modulate EGFR accessibility to external stimuli and thus contribute to regulate its function in cell proliferation and differentiation processes. It can also provide new sources of alterations leading to tumorsgenesis, as well as new targets for antitumoral therapies.

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Vol. 21, August 15, 2010 2929