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

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**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.**

## INTRODUCTION

Endocytosis plays a predominant role among the variety of mechanisms that regulate the function of epidermal growth factor receptor (EGFR; Yarden and Sliwkowski, 2001; Sorkin and Goh, 2009). EGFR activated by ligand binding is rapidly endocytosed and can recycle remaining active during variable periods of time before entering into the lysosomal-degradation route for down-regulation. Endocytic trafficking expands the opportunities to modulate outcome responses providing mechanisms to compartmentalize, increase or attenuate signals, adding space and time dimensions (Scita and Di Fiore, 2010). An important questions is whether endocytosis might also regulate the distribution of empty/inactive EGFR between the cell surface and intracellular compartments, and thus its accessibility to extracellular stimulus, in response to heterologous signaling, as suggested by several studies (Salazar and Gonzalez, 2002; Vergarajauregui *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-quinase 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 (Madshus and Stang, 2009; Sorkin and Goh, 2009). Depending on the kind of ligand (e.g., EGF or TGF-$\alpha$), and their concentration, the endocytic route taken by activated EGFR varies according to receptor ubiquitination levels (Sigismund *et al.*, 2005, 2008;

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Abbreviations used: EGF, epidermal growth factor; EGFR, EGF receptor; EEA1, early endosome antigen 1; CHC, clathrin heavy chain; DAG, diacylglycerol; diTf, diferric Tf; FIPI, 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.

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

Less known regulatory mechanisms operate upon empty/inactive EGFR. In the absence of ligand, phorbol esters have been shown to induce transient internalization of EGFR, suggesting a pathway regulated by protein kinase C (Beguinot *et al.*, 1985). Internalization of empty/inactive EGFR can also be induced by activation of p38 mitogen-activated protein kinase (Vergarajauregui *et al.*, 2006; Winograd-Katz and Levitzki, 2006; Zwang and Yarden, 2006) or by inhibition of protein kinase A (PKA) basal activity (Salazar and Gonzalez, 2002). Therefore, signaling pathways emerging from a variety of receptors might hypothetically transmodulate the permanency 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 involve in membrane trafficking (Roth, 2008; Donaldson, 2009) and signaling from a variety of receptors (Liscovitch *et al.*, 2000; Wang *et al.*, 2006), including EGFR (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.*, 2001; Lee *et al.*, 2006, 2009). In signal transduction, PLD activity is increased by a large variety of stimulus and receptor pathways (Liscovitch *et al.*, 2000; Wang *et al.*, 2006; Zhao *et al.*, 2007) and the generated PA recruits and modulates signaling elements bearing PA-binding domains, such as Sos and Raf (Stace and Ktistakis, 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; Asp *et al.*, 2009), provokes a reversible redistribution of empty/inactive EGFR from the cell surface to recycling endosomes through the PA-mediated signaling toward the PDE4/cAMP/PKA pathway, modulating receptor accessibility to external stimulus.

## MATERIALS AND METHODS

### *Reagents and Antibodies*

We used human recombinant EGF from Invitrogen (Carlsbad, CA), Protein A-Sepharose, propranolol, and DMEM 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 Nalgen Nunc (Naperville, IL). Polyclonal antibody EGFR984 against EGFR cytosolic residues 984–996 has been previously characterized (Faúndez *et al.*, 1992). Hybridomas producing monoclonal anti-EGFR antibodies HB8506 against the EGFR 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. 610500; Lexington, KY). Noncommercial reagents include diferric transferrin (diTf), prepared from apoTf as described (Bali and Harris, 1990; provided by Dr. Tulio 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 (FIPI; Su *et al.*, 2009; provided by Dr. Michael Frohman, Stony Brook University, NY).

### *Cell Culture*

Cells previously characterized for EGFR 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 EGFR (Her14 cells) or a point mutant kinase-negative EGFR (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 EGFR, and rat neuroblastoma N2a cells permanently transfected with EGFR or with FLAG-tagged $\mu$-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 $CaCl_2$ and 1 mM $MgCl_2$ (PBS-CM). After washing three times with PBS plus 0.2% gelatin (300 Bloom; 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-EGFR mAb (mAb) HB8506 (1/100 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 EGFR 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 three times with ice-cold PBS and then incubated 2 h in DMEM-HEPES serum-free media at 37°C to accumulate EGFR on the cell surface. Serum-deprived cells were then preincubated with 50 $\mu$g/ml Tf-Alexa594 or 1 $\mu$M lysotracker at 37°C for 1 h and then with 10 ng/ml EGF or 100 $\mu$M propranolol for the indicated time periods. Digital fluorescence images were acquired on a Zeiss Axiophot microscope with a Plan-APOCHROMAT 63×/1.4 oil immersion objective and the 14-bit Zeiss Axiocam 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 EGFR overlap with EEA1, Tf-Alexa594 or lysotracker was calculated for each individual cell (n = 30–40 cells).

### *Flow Cytometry*

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

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 conditions that induce endocytosis. After acid wash at 4°C to remove 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).

### *Ligand-binding Assays and Endocytic Rate Constants*

Human $^{125}$I-EGF was prepared by the chloramine T method as described (Faúndez *et al.,* 1992), obtaining specific activities of 50,000–70,000 cpm/ng. Binding assays were done as described (Salazar and Gonzalez, 2002) in Hanks' solution with 20 mM HEPES and 0.1% bovine serum albumin (BSA) during 2 h at 4°C. diTf was iodinated with IODO-GEN following the manufacturer's specifications (Pierce Biotechnology, Rockford, IL), achieving a specific activity of 30,000 cpm/ng. Cells were incubated with 10 ng/ml $^{25}$I-diTf for 2 h at 4°C. After washing the unbound ligand, the cells were incubated at 37°C for 5–10 min and surface-bound, and internalized ligand determined by acid-wash (Salazar and Gonzalez, 2002) was used to estimate the endocytic rate constant (ke) by the IN/SUR method, as the slope of the plot of the ratio of internalized (IN)-to-surface (SUR) ligand versus time (Wiley and Cunningham, 1982).

### *Cell Surface Biotinylation, Immunoprecipitation, and Immunoblot Assays*

To analyze separately the EGFR endocytic and postendocytic return to the cell surface, we used biotinylation with the reducible agent sulfo-NHS-SS-biotin, as described (Salazar and Gonzalez, 2002; Burgos *et al.,* 2004). Detection of EGFR tyrosine phosphorylation and ubiquitination was also as described (Salazar and Gonzalez, 2002). Cell protein was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA) using BSA as standard. EGFR was isolated from equivalent total protein of cell lysates by precipitation with Immobilized Neutravidin or by immunoprecipitation with the mAb HB8506 previously bound to 30 $\mu$l of protein A-Sepharose CL-4B beads per sample (Salazar and Gonzalez, 2002). The beads were washed six times in ice-cold buffer (20 mM HEPES, 150 mM NaCl, 10% glycerol, and 0.1% Triton X-100) for 5 min each and incubated in 20 $\mu$l of sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 100 mM dithiothreitol (DTT), 10% glycerol, and 0.005% bromophenol blue) for 5 min at 65°C. Eluates were run in a 7.5% SDS-PAGE, and the proteins were electrotransferred onto nitrocellulose filters. Immunoblots made with polyclonal antibody against EGFR (Chemicon, Temecula, CA) or the monoclonal anti-phosphotyrosine 4G10 or anti-ubiquitin P4D1 antibodies were revealed with ECL (Amersham Biosciences; Salazar and Gonzalez, 2002) and the bands were digitalized in a VISTA-T630 UMax scanner driven by Adobe Photoshop CS (Adobe Systems, Mountain View, CA).

### *PDE, cAMP and PKA Assays*

The method of Thompson and Appleman (1971) was used to measure PDE activity. Briefly, samples were assayed in a reaction mixture of 200 $\mu$l containing 40 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM $\beta$-mercaptoethanol, 1 $\mu$M cAMP, 0.75 mg/ml BSA, and 0.1 $\mu$Ci [$^3$H]cAMP for 10 min at 33°C. The reaction was stopped by adding 200 $\mu$l 10 mM EDTA in 40 mM Tris-HCl, pH 8.0, followed by heat inactivation in a boiling water bath for 1 min. The PDE reaction product 5′-AMP was hydrolyzed by incubation of the assay mixture with 50 $\mu$g of *Crotalus atrox* snake venom for 20 min at 33°C, and the resulting adenosine was separated by anion exchange chromatography using 1 ml of AG1-X8 resin and quantified by scintillation counting in a LKB Wallac 1217 Rackbeta liquid scintillation counter (Turku, Finland). cAMP was assessed with the TRK 432 kit (Amersham) and PKA activity with the SignaTECT kit (Promega) according to manufacturer's recommendations.

### *PA Measurements and Micelles Preparation*

PA levels were estimated as described (Tomic *et al.,* 1995). Briefly, HeLa cells were grown in six-well dishes in DMEM 7.5% SFB to 80% of confluence, incubated with 10 $\mu$Ci [9,10-$^3$H]myristic in DMEM 0.3% SFB overnight, and organic phase extraction was made by adding 300 $\mu$l of cold MetOH:HCl (50:2), 600 $\mu$l of chloroform, and 200 $\mu$l 1 M NaCl. The organic phase was evaporated under nitrogen flow, and the pellet was resuspended in 60 $\mu$l chloroform: methanol (90:10). Lipids of a 20-$\mu$l sample were loaded in a thin-layer chromatography (TLC) plate (Merck, Darmstadt, Germany) and separated in chloroform:acetone:methanol:acetic acid:water (40:15:15:12:7.5) mixture for 2 h at room temperature. PA standard was visualized with iodine crystals. TLC pretreated with ENHANCER was developed by autoradiography using Hyperfilm (Amersham, GE Healthcare, Amersham, United Kingdom). PA micelles were prepared as described (Tomic *et al.,* 1995). Briefly, a solution of PA (3-sn-PA from Sigma) dissolved in 20 mM imidazole, pH 7.0, 1 mM EDTA, and 1 mM DTT was placed in a bath-sonifier (ultrasons-H Selecte) for 15 min twice.

### *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 $\mu$g of plasmid for 6 h or 0.3 $\mu$g for 18 h. Only cells with normal morphology were analyzed. Small interference RNAs (siRNAs) were purchased from Dharmacon (Boulder, CO), using the commercial on-target plus smart pool L-004001-01-0005 for clathrin heavy chain (CHC; Gene accession number NM__004859) and L-009413-00-0005 for PLD1 (Gene accession number NM__002662). For PLD2 we used the described sequence (Padron *et al.,* 2006) aagagguggcuggugguguuguu (Gene accession number NM__002663). Controls siRNA were siGENOME nontargeting siRNA 2 (D-001210-02-05) and 4 (D-001210-04-05). HeLa cells grown to 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, caactacagaaccatgtgcag; reverse, AATGACAGCCATTTCACTGTC; and PLD2: forward, AAAGATATACCAGCGGATCC; reverse, CAGGTATTTCTGTTTGCTGG. Expression of CHC was analyzed by immunoblot with a mAb (BD Transduction Laboratories) on 50 $\mu$g 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 $^{125}$I-EGF binding activity, up to ~80% at 250 $\mu$M, with half maximal effective concentration of ~75–100 $\mu$M, depending on the propranolol batch (Figure 1A, left). It is very likely that propranolol at 250 $\mu$M almost completely inhibited the PAP activity (Asp *et al.,* 2009). Higher concentrations provoked cell detachment. We then used concentrations of 75–100 $\mu$M for the rest of the experiments. Time-course experiments with 100 $\mu$M propranolol showed that $^{125}$I-EGF binding decreases with $t_{1/2}$ ~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 $t_{1/2} \sim 5$ 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 population of HeLa cells express ~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.,*

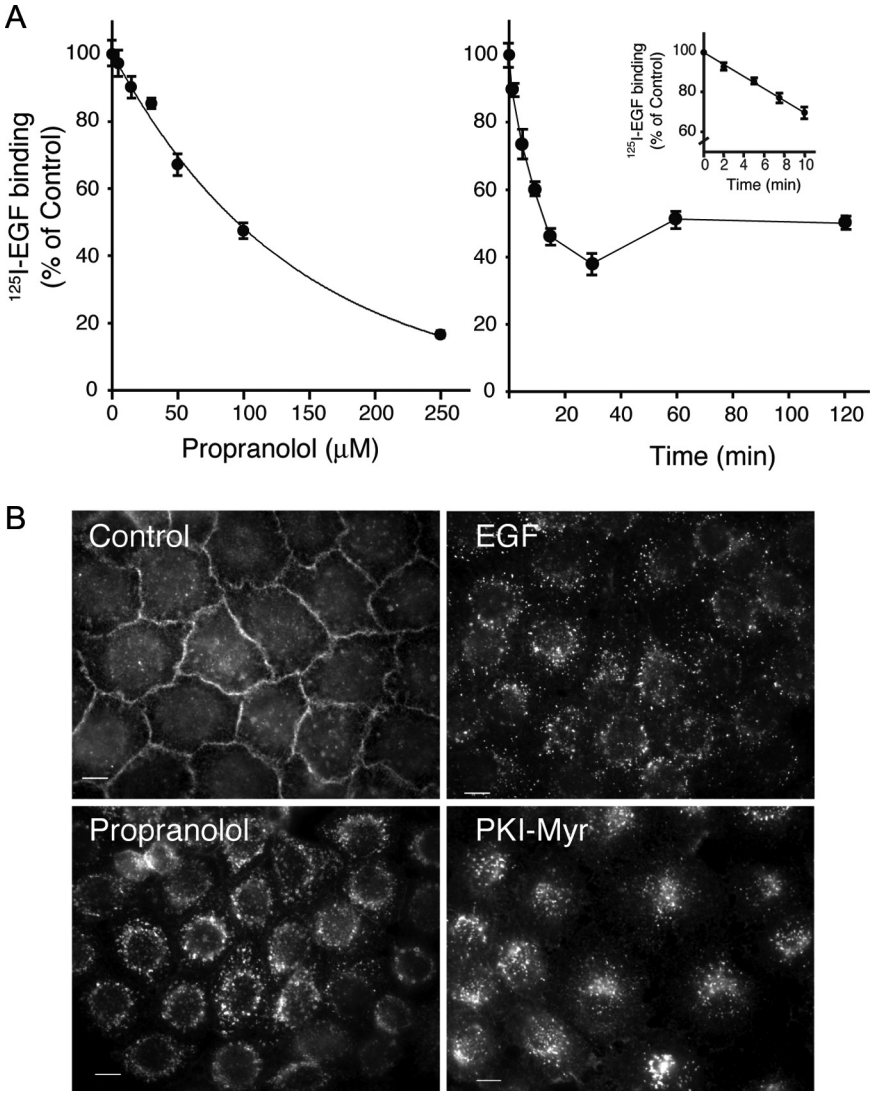


**Figure 1.** PAP inhibition by propranolol induces internalization of EGFR, mimicking the effect of PKA inhibitors. (A) Dose-dependent effect of propranolol on $^{125}$I-EGF binding (left). Right panel shows time course with 100 $\mu$M propranolol, and the inset shows in detail the kinetics of decrease 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 $\mu$M), or propranolol (75 $\mu$M) 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 $\mu$m.

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 stimulus (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 $\mu$M) 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

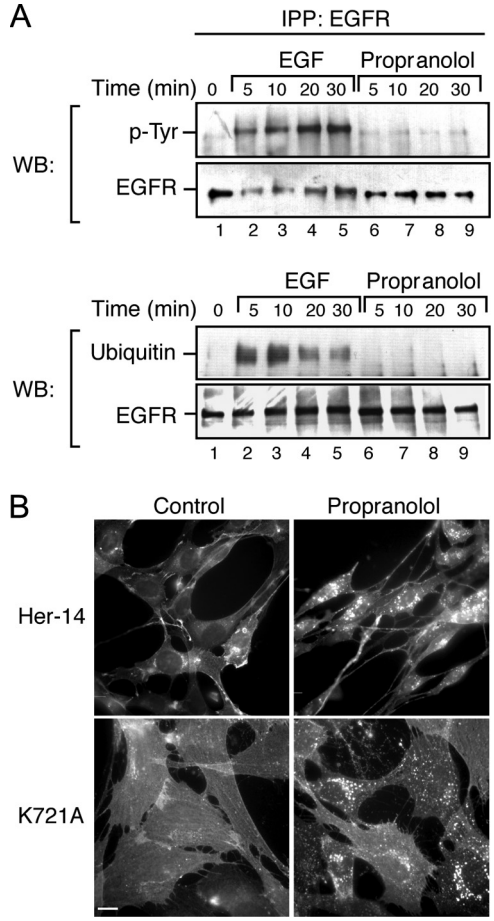


**Figure 2.** Under propranolol treatment, EGFR is not transactivated and is internalized independently of its tyrosine-kinase activity. (A) Propranolol does not induce tyrosine phosphorylation or ubiquitination of EGFR. HeLa cells were incubated in the absence (lane 1) or presence of either 50 ng/ml EGF (lanes 2–5) or 75 $\mu$M 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 $\mu$M) 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 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 $^{125}$I-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 $\mu$g/ml) for 30 min at 37°C. This treatment decreased $^{125}$I-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.*, 1990).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 $^{125}$I-EGF–binding activity elicited by propranolol was now reduced to only ~10–15% in the presence of 30 $\mu$M 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 adenylylcyclases by forskolin and overexpressing the catalytic subunit of PKA. Forskolin inhibited ~70% of the EGFR internalization induced by propanol (Figure 4F). The experiments of PKA overexpression were performed adjusting the conditions to avoid expected toxic effects. Transfections with either 1 $\mu$g of PKA-YFP vector for 6 h or 0.3 $\mu$g 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 PA 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

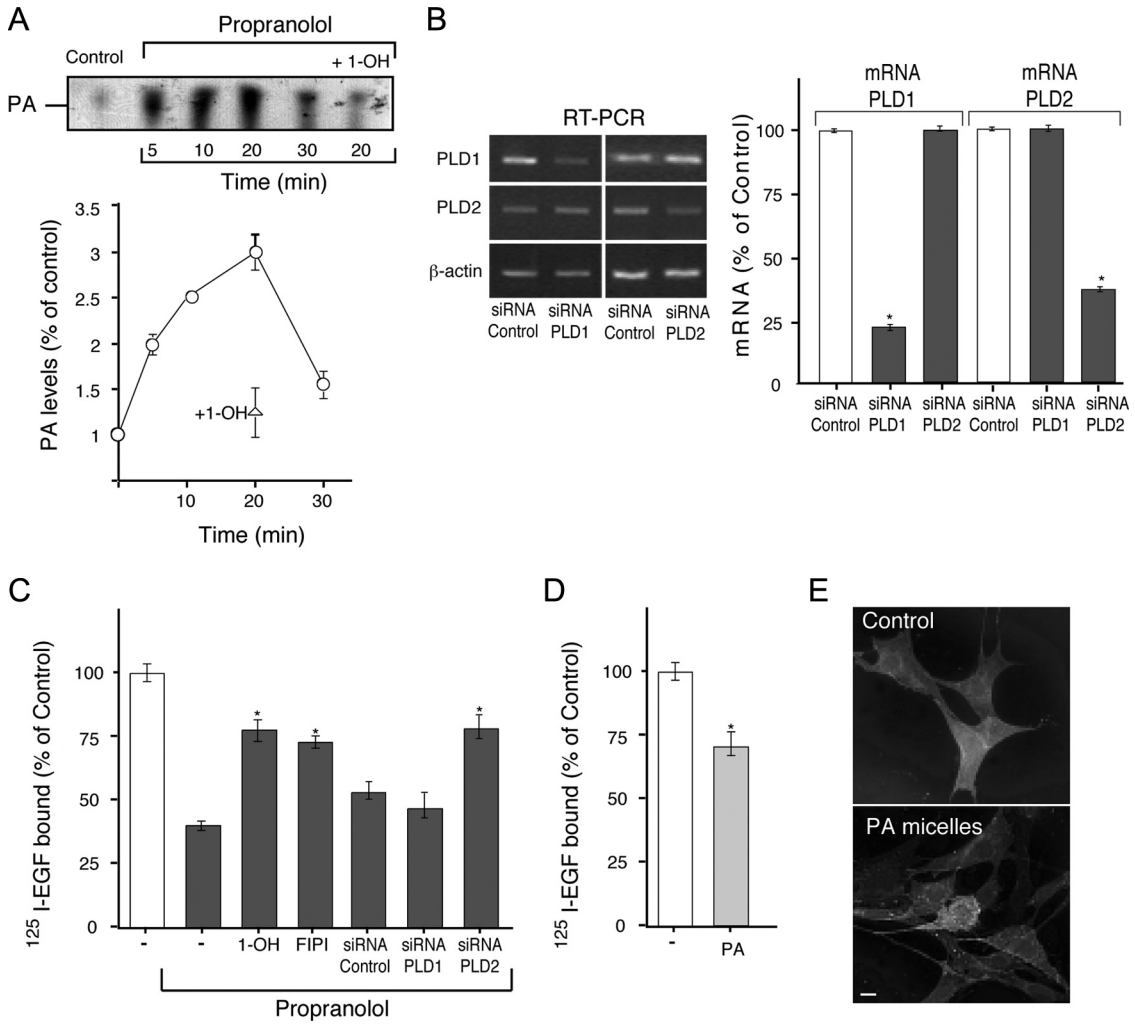


**Figure 3.** PA generated by PLD2 mediates EGFR internalization induced by propranolol. (A) PAP inhibition by propranolol provokes a transient increase of PA levels. HeLa cells were pulse-labeled with [$^3$H]myristic acid (10 $\mu$Ci) for 16 h and then treated with 75 $\mu$M propranolol for 30 min at 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) Counteracting effects of PLD inhibition or silencing. The decrease in $^{125}$I-EGF binding induced by 100 $\mu$M propranolol (100% effect) is counteracted ~50% by FIPI, 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 $^{125}$I-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.

internalization rate of Tf, which is constitutively endocytosed via the clathrin pathway (Maxfield and McGraw, 2004), nor the distribution of the $\mu$-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 $^{125}$I-Tf endocytosis showed an endocytic constant (ke) of 0.12 ± 0.013 min$^{-1}$ that did not significantly change in propranolol-treated cells (ke = 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 ($t_{1/2}$ ~20–30 min) and a predominant fast component ($t_{1/2}$ ~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

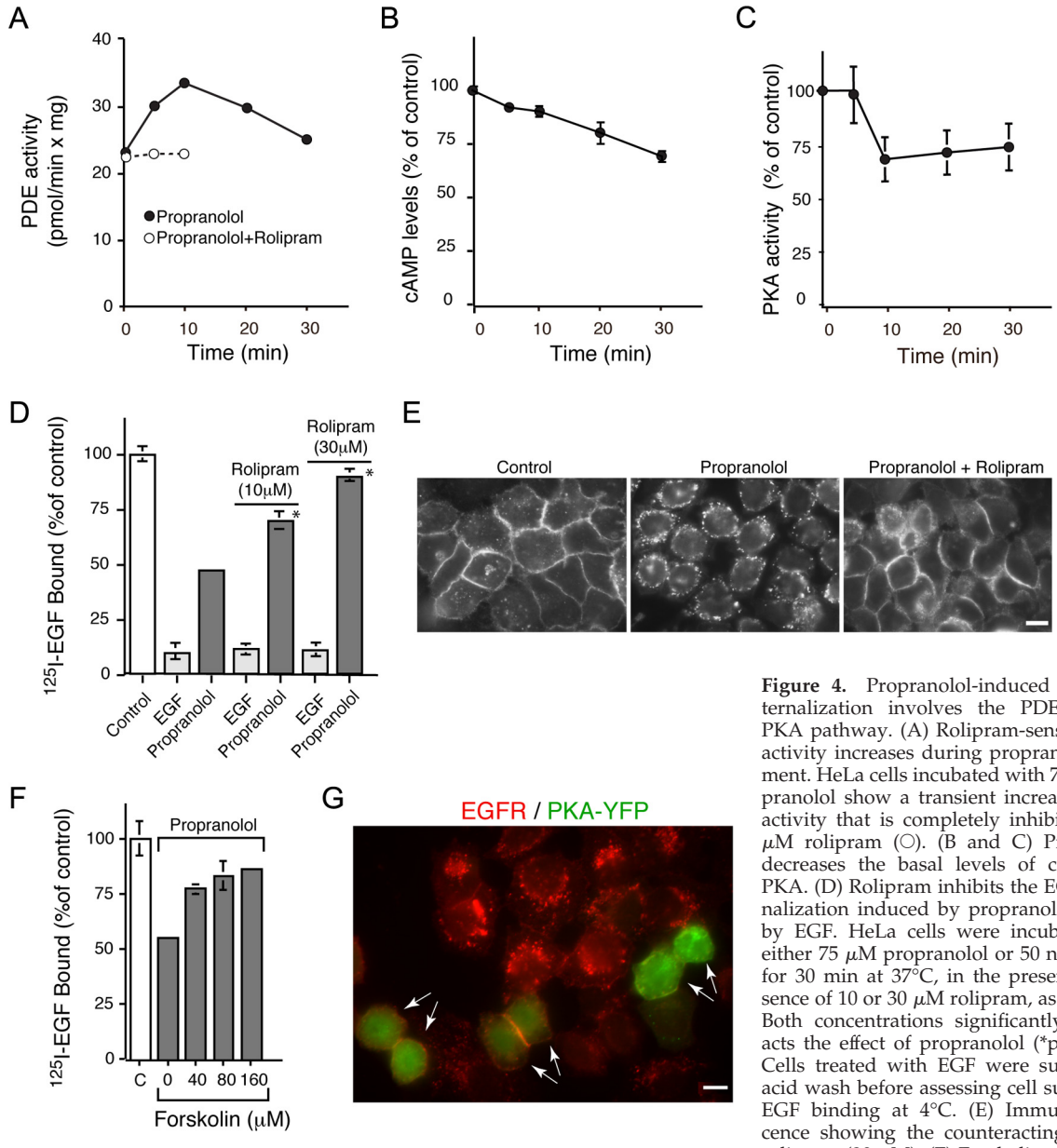


**Figure 4.** Propranolol-induced EGFR internalization involves the PDE4/cAMP/PKA pathway. (A) Rolipram-sensitive PDE activity increases during propranolol treatment. HeLa cells incubated with 75 $\mu$M propranolol show a transient increase in PDE activity that is completely inhibited by 30 $\mu$M rolipram (○). (B and C) Propranolol decreases the basal levels of cAMP and PKA. (D) Rolipram inhibits the EGFR internalization induced by propranolol but not by EGF. HeLa cells were incubated with either 75 $\mu$M propranolol or 50 ng/ml EGF for 30 min at 37°C, in the presence or absence of 10 or 30 $\mu$M rolipram, as indicated. Both concentrations significantly counteracts the effect of propranolol (*$p < 0.001$). Cells treated with EGF were subjected to acid wash before assessing cell surface $^{125}$I-EGF binding at 4°C. (E) Immunofluorescence showing the counteracting effect of rolipram (30 $\mu$M). (F) Forskolin counteracts the EGFR internalization induced by propranolol. Cells were preincubated with the indicated concentrations of forskolin for 30 min and then treated with propranolol for another 30 min (*$p < 0.001$). (G) Overexpression of PKA catalytic subunit abrogates the endocytic effect of propranolol. HeLa cells were transfected with 1 $\mu$g PKA-YFP (green) plasmid for 6 h and then incubated with 100 $\mu$M propranolol for 30 min and treated for indirect immunofluorescence with anti-EGFR monoclonal antibodies (red). Most of the cells (>97%) expressing PKA-YFP (arrows) do not display internalized EGFR, contrasting with most of the cells not expressing PKA-YFP. Merged image; Bar, 10 $\mu$m.

suggested by the rate of ligand-binding decrease ($t_{1/2}$ ~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

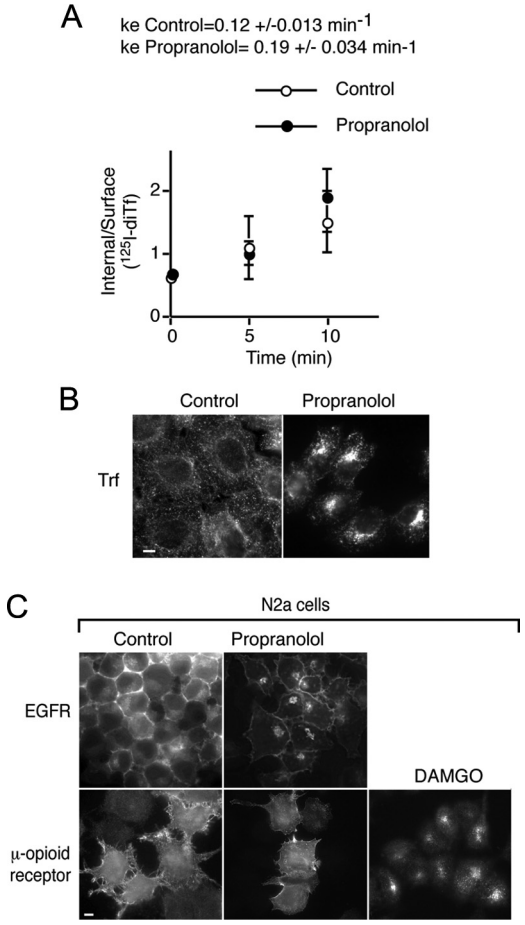


**Figure 5.** Propranolol does not affect endocytosis of TfR and $\mu$-opioid receptors, but promotes intracellular accumulation of Tf-Alexa 549. (A) Constitutive endocytosis of TfR. HeLa cells were preincubated in serum-free media for 1 h at 37°C and then with 20 ng/ml $^{125}$I-diTf for 2 h at 4°C. After eliminating the unbound $^{125}$I-diTf, cells were incubated in the presence or absence of 75 $\mu$M 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 $\mu$g/ml Tf-Alexa 549 at 37°C for 30 min in the absence or presence of 75 $\mu$M and fixed for fluorescence imaging show strong juxtanuclear fluorescence accumulation, suggesting decreased TfR recycling. (C) Propranolol treatment does not cause redistribution of $\mu$-opioid receptors. N2a cells permanently transfected with either EGFR or FLAG-tagged $\mu$-opioid receptors were incubated with either 75 $\mu$M propranolol or 10 $\mu$M DAMGO ($\mu$-opioid receptor ligand), for 30 min. Bar, 10 $\mu$m.

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 $\mu$M) treatment decreased the $^{125}$I-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 nonselective 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.

### *The PA/PKA Pathway Leads to EGFR Accumulation in Recycling Endosomes Escaping Degradation for Several Hours*

EGFR represents the paradigm of ligand-induced endocytosis leading to down-regulation by intracellular targeting into the lysosomal degradation pathway (Wiley, 2003; Sorkin and Goh, 2009). Recent evidence shows that EGFR activated with low ligand concentrations (1–2 ng/ml) stimulates a recycling route associated with lack of receptor ubiquitination, whereas high concentrations of EGF (10–100 ng/ml) promote receptor entry into both clathrin-dependent and -independent pathways, leading to degradation associated with receptor ubiquitination (Sigismund *et al.*, 2005, 2008). We compared the fate of internalized EGFR during stimulation with high EGF concentration (10–50 ng/ml) versus that induced by propranolol treatment. Cells incubated with 50 ng/ml EGF showed the expected decrease in the mass of EGFR, with $t_{1/2} \sim 60$ min, whereas under propranolol treatment the receptor remained constant for up to 4 h (Figure 7A). Together with the results of Figure 1B, this result suggests that empty/inactive EGFR accumulates in endocytic compartments diverging from the lysosomal degradation pathway.

To assess the intracellular fate of internalized EGFR, we performed quantitative colocalization analysis using established markers for early and late endosomes/lysosomes. Early endosomes include peripherally sorting endosomes and juxtanuclear recycling endosomes, both loadable with Tf, whereas only sorting endosomes contain EEA1 (Maxfield and McGraw, 2004). Late endosomes and lysomes can be easily detected with lysotraker. The intracellular location of EGFR internalized during treatment with either 10 ng/ml EGF or 100 $\mu$M propranolol

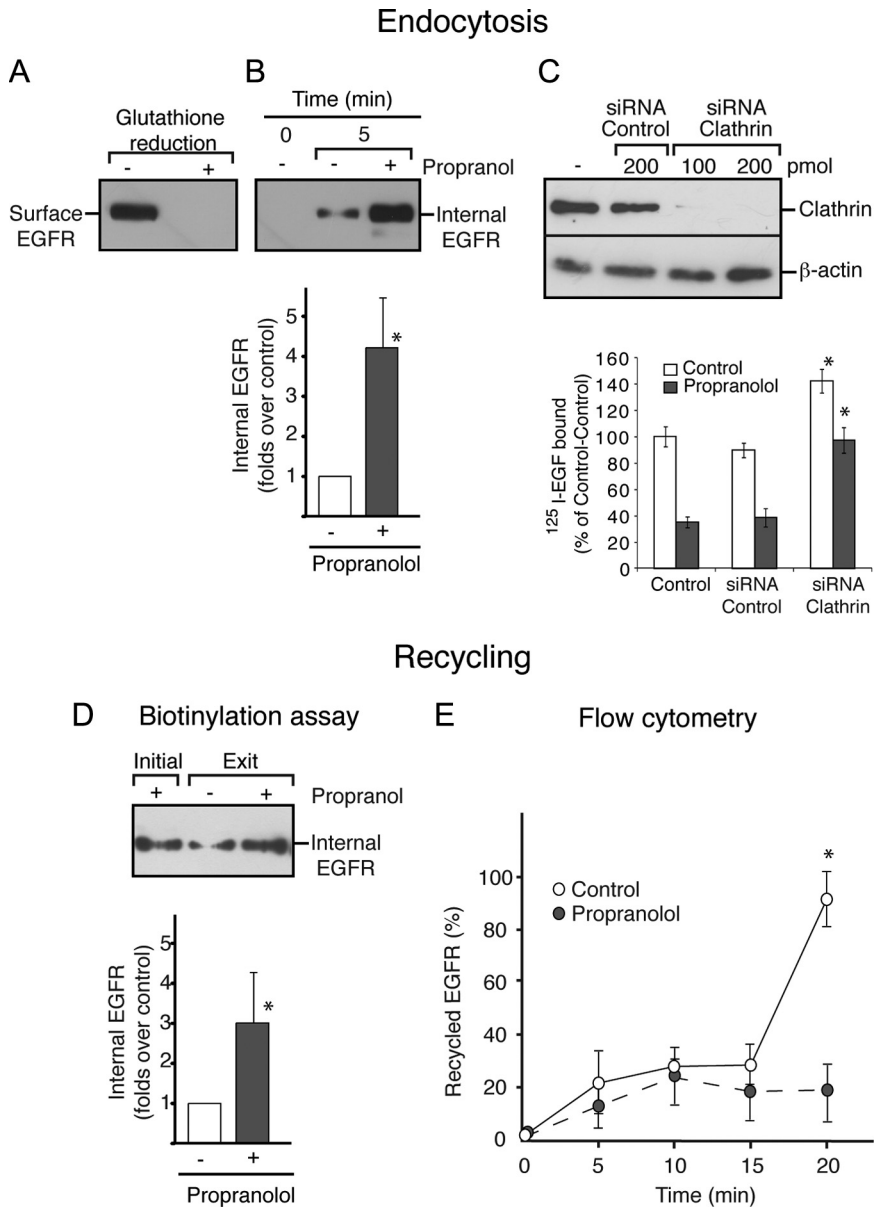


**Figure 6.** Propranolol increases endocytosis (clathrin-dependent and -independent) and inhibits recycling of empty EGFR. (A and B) Endocytosis of cell surface biotinylated EGFR. Cells were biotinylated with cleavable EZ-Link sulfo-NH-SS-biotin at 4°C and then incubated for 5 min at 37°C in the absence (−) or presence (+) of 75 $\mu$M propranolol. Glutathione reduction efficiently removes the biotin from the cell surface EGFR (A, and time 0 in B) and reveals a three- to fourfold higher mass of resistant (internalized) EGFR under the effect of propranolol (B), quantified by densitometry (average ± SEM; n = four experiments; *$p < 0.05$). (C) Top, effect of clathrin silencing with siRNA on EGFR endocytosis. HeLa cells transfected twice, each 24 h, with CHC siRNA show almost complete abrogation (>95%) of clathrin expression, as shown by immunoblot 72 h after the first transfection. Bottom, graph shows the effect of propranolol (100 $\mu$M) on $^{125}$I-EGF–binding activity in cells transfected with control or clathrin siRNA. Clathrin silencing counteracts only partially (~50%) this effect, suggesting that a clathrin-independent pathway is also involved (average ± SEM; n = three experiments in triplicate; *$p < 0.001$). (D) Postendocytic cell surface return of previously internalized biotinylated EGFR. Cells biotinylated with cleavable EZ-Link sulfo-NH-SS-biotin at 4°C were treated for 30 min with propranolol at 37°C, shifted to 4°C, and stripped with glutathione (Initial, internalized pool). A second incubation for another 30 min at 37°C in the absence (−) or presence (+) of 100 $\mu$M propranolol followed by glutathione treatment shows three- to fourfold more intracellular EGFR (resistant to glutathione) under the effect of propranolol (graph, average ± SEM; n = four experiments; *$p < 0.05$). (E) Flow cytometry analysis of cell surface return of previously internalized antibody-EGFR complex. Cells incubated at 4°C with mAb against extracellular EGFR were treated with 100 $\mu$M propranolol for 30 min, acid washed at 4°C to remove the remaining cell surface antibodies, and then placed at 37°C for the indicated time periods, in the absence (Control) or presence of propranolol. After incubation with fluorescent secondary antibody, flow cytometry analysis was performed. Progressive cell surface reappearance of EGFR is similar within the first 10–15 min, but afterward ~90% of EGFR returns to the cell surface in the absence of propranolol (average ± SEM; n = three experiments; *$p < 0.05$).

was compared (Figure 7, B and C). HeLa cells were first incubated for 1 h with either 50 $\mu$g/ml Tf-Alexa 594, to load sorting and recycling endosomes, or with 1 $\mu$M lysotraker, 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 TfR, 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

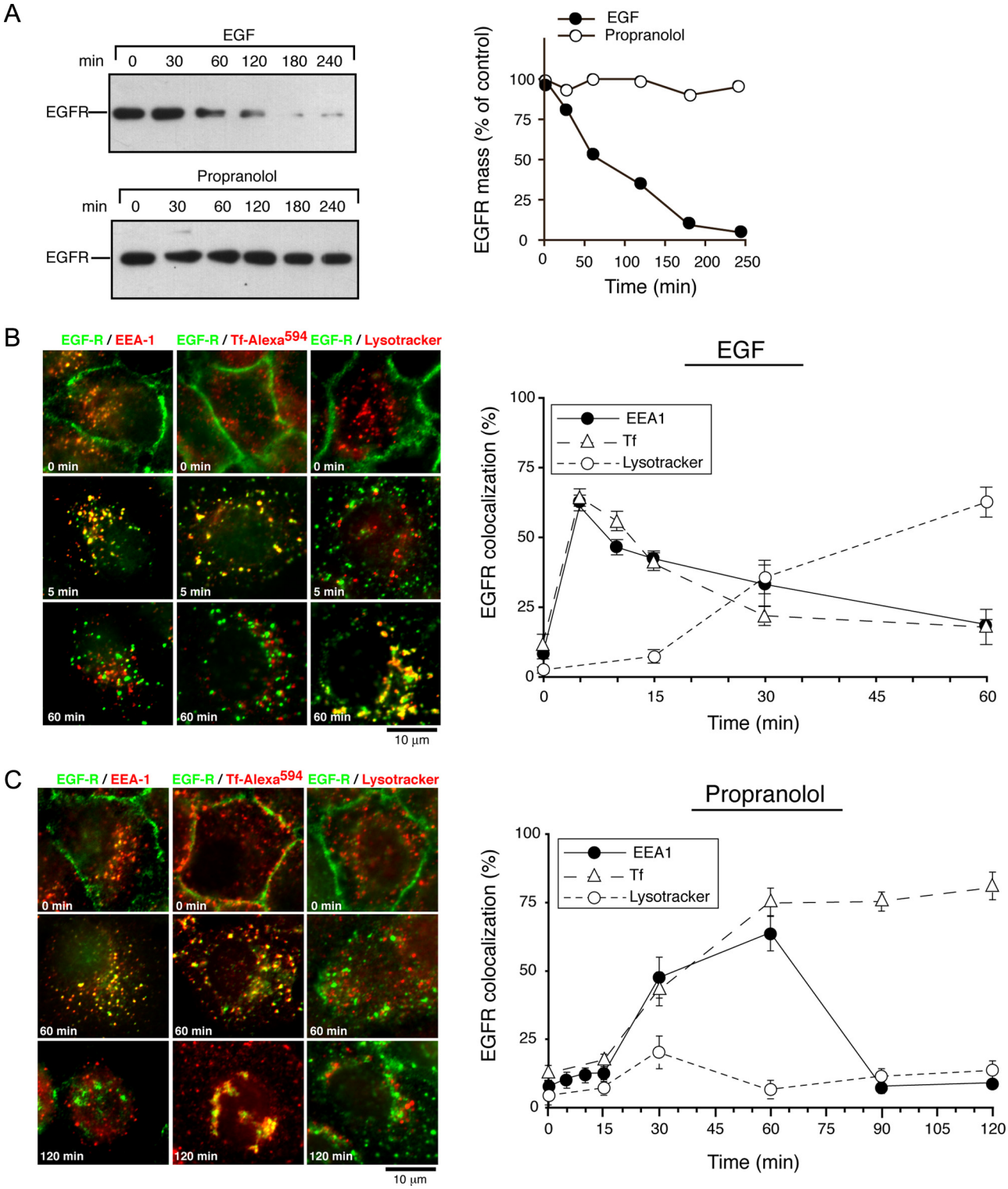


**Figure 7.** EGFR internalized during propranolol treatment accumulates in recycling endosomes without degradation. (A) EGFR mass under EGF or propranolol treatment. Immunoblots with polyclonal antibody EGFR984 and densitometric analysis (graph) show an almost unchanged EGFR mass for up to 4 h of propranolol (75 $\mu$M) treatment, instead of the progressive decrease ($t_{1/2}$ ~60 min) seen during incubation with EGF (50 ng/ml). (B and C) Immunofluorescence colocalization analysis of internalized EGFR. HeLa cells preincubated with 50 $\mu$g/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 $\mu$M 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 $\mu$m.

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 TfR.

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 TfR (Padron *et al.*, 2003). However, we did not observe an accelerated rate of TfR endocytosis, suggesting that the increment in PA levels produced by the low propranolol concentrations used in our experiments (75–100 $\mu$M) 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. $\mu$-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 (Gorodinsky 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 internalized 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 TfR recycling also occurs under propranolol treatment. Membrane recycling of cargo endocytosed via clathrin-independent pathways seems to depend on Arf6-mediated acti-

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 TfR 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 adenylylcyclases 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 (Vergarajauregui *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 $\mu$-opioid (Koch *et al.*, 2004), constitutive endocytosis of the metabotropic glutamate receptor (Bhattacharya *et al.*, 2004), constitutive recycling but not endocytosis of TfR (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 $\mu$2 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 tumorigenesis, as well as new targets for antitumoral therapies.

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