

# Gq-coupled Purinergic Receptors Inhibit Insulin-like Growth Factor-I/Phosphoinositide 3-Kinase Pathway-dependent Keratinocyte Migration

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**Insulin-like growth factor-I (IGF-I) activation of phosphoinositide 3-kinase (PI3K) is an essential pathway for keratinocyte migration that is required for epidermis wound healing. We have previously reported that activation of  $G_{\alpha(q/11)}$ -coupled  $P2Y_2$  purinergic receptors by extracellular nucleotides delays keratinocyte wound closure. Here, we report that activation of  $P2Y_2$  receptors by extracellular UTP inhibits the IGF-I-induced p110 $\alpha$ -PI3K activation. Using siRNA and pharmacological inhibitors, we demonstrate that the UTP antagonistic effects on PI3K pathway are mediated by  $G_{\alpha(q/11)}$ —and not  $G_{(i/o)}$ —independently of phospholipase C $\beta$ . Purinergic signaling does not affect the formation of the IGF-I receptor/insulin receptor substrate-1/p85 complex, but blocks the activity of a membrane-targeted active p110 $\alpha$  mutant, indicating that UTP acts downstream of PI3K membrane recruitment. UTP was also found to efficiently attenuate, within few minutes, the IGF-I-induced PI3K-controlled translocation of the actin-nucleating protein cortactin to the plasma membrane. This supports the UTP ability to alter later migratory events. Indeed, UTP inhibits keratinocyte spreading and migration promoted by either IGF-I or a membrane-targeted active p110 $\alpha$  mutant, in a  $G_{\alpha(q/11)}$ -dependent manner both. These findings provide new insight into the signaling cross-talk between receptor tyrosine kinase and  $G_{\alpha(q/11)}$ -coupled receptors, which mediate opposite effects on p110 $\alpha$ -PI3K activity and keratinocyte migration.**

## INTRODUCTION

Skin lesions initiate the release of various growth factors in the wound bed, cytokines, and low-molecular-weight soluble factors such as extracellular nucleotides (Werner and Grose, 2003). Together, these factors orchestrate a cascade of events leading to a proper wound healing, which includes reepithelialization. During this step, basal keratinocytes, i.e., the epidermis epithelial cells, undergo morphological changes required for their migration from the wound margin over the denuded area (Patel *et al.*, 2006; Hosoya *et al.*, 2008). Insulin-like growth factor-I (IGF-I), acting through paracrine/autocrine regulatory loops, is a peptide growth factor mainly secreted by dermal cells (macrophages and fibroblasts) but also by keratinocytes (although controversy remains regarding keratinocyte IGF-I production; Edmondson *et al.*, 2003). Its biological function is mediated by its

cognate tyrosine kinase receptor (IGF-IR), which is expressed by basal keratinocytes (Hodak *et al.*, 1996; Sadagurski *et al.*, 2006). The involvement of IGF-I in wound healing is supported by its up-regulation after wounding and the lack of expression in nonhealing wound (Edmondson *et al.*, 2003). Moreover, gene-mediated IGF-I expression by basal keratinocytes in the wound bed increases the rate of reepithelialization (Jeschke *et al.*, 2005; Semenova *et al.*, 2008; Todorovic *et al.*, 2008). IGF-I is also a strong promoter of *in vitro* keratinocyte migration (Ando and Jensen, 1993; Haase *et al.*, 2003; Sadowski *et al.*, 2003; Semenova *et al.*, 2008). Thus, an increase of keratinocyte migration may partly explain the acceleration of wound reepithelialization mediated by IGF-I (Semenova *et al.*, 2008).

The first step in the signal transduction by IGF-IR is its autophosphorylation followed by the subsequent recruitment and the extensive tyrosine phosphorylation of insulin-receptor substrate-1 (IRS-1), which then initiates several distinct signaling pathways including phosphatidylinositol 3-kinase (PI3K; Baserga, 2000). Once recruited to the plasma membrane, PI3K phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP<sub>2</sub>) to produce phosphatidylinositol (3,4,5)-trisphosphate (PIP<sub>3</sub>), which serves as a docking signal for the pleckstrin homology (PH) domain-carrying proteins, such as the protein kinase B/Akt (Akt). Class IA PI3Ks are heterodimers composed of a p110 catalytic subunit (three isoforms:  $\alpha$ ,  $\beta$ , and  $\delta$ ) and a smaller regulatory subunit (five different isoforms: p85 $\alpha$ , p85 $\beta$ , p55 $\alpha$ , p50 $\alpha$ , and p55 $\gamma$ ; Hawkins *et al.*, 2006) and are among of the most important proteins that control cell migration (Merlot and Firtel, 2003; Cain and Ridley, 2009). In the epidermis, keratinocytes

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Abbreviations used: GPCR, G protein-coupled receptor; IGF-I, insulin-like growth factor-I; PI3K, phosphoinositide 3-kinase; PIP<sub>2</sub>, phosphatidylinositol (4,5)bisphosphate; PIP<sub>3</sub>, phosphatidylinositol (3,4,5) trisphosphate; PLC $\beta$ , phospholipase C  $\beta$ ; P2Y<sub>2</sub>R, P2Y<sub>2</sub> receptor; RTK, receptor tyrosine kinase.

strongly express p110 $\alpha$  and p110 $\beta$  (Pankow *et al.*, 2006), and the expression of an active p110 $\alpha$  mutant is sufficient to promote lamellipodium formation, cell spreading, and cell migration in vitro (Haase *et al.*, 2003; Pankow *et al.*, 2006). Furthermore, in murine skin biopsies, pharmacological inhibition of PI3K suppresses keratinocyte migration (Pankow *et al.*, 2006).

As underlined above, extracellular nucleotides (ATP, UTP) are released in the wound bed by many different cell types including platelets and keratinocytes (Lazarowski *et al.*, 2003; Holzer and Granstein, 2004). It has been reported that damaged cultured keratinocytes can release ATP at micromolar concentration, which is sufficiently high to activate their cognate receptors (Burrell *et al.*, 2005; Yin *et al.*, 2007). ATP and UTP as other extracellular nucleotides exert their function by activating two classes of purinergic receptors, the G-protein-coupled receptors (P2YRs) and the ATP-gated ion channels (P2XR<sub>s</sub>; Burnstock, 2006). In the epidermis, basal keratinocytes mainly express P2Y<sub>1</sub>R and P2Y<sub>2</sub>R (Dixon *et al.*, 1999; Greig *et al.*, 2003a). After wound, the expression and distribution of P2Y<sub>1</sub>R and P2Y<sub>2</sub>R is modulated, indicating that these receptors may participate to the healing process (Greig *et al.*, 2003b). In keratinocytes, the ATP/UTP-sensitive G $\alpha_q$ -coupled P2Y<sub>2</sub>R was found to be the main functional receptor activated by UTP (Lee *et al.*, 2001; Koizumi *et al.*, 2004; Yoshida *et al.*, 2006; Inoue *et al.*, 2007). Moreover, a growing body of evidence supports that P2Y<sub>2</sub>R is an important mediator of cell migration in several cell types (Chaulet *et al.*, 2001; Pillois *et al.*, 2002; Bagchi *et al.*, 2005; Kaczmarek *et al.*, 2005; Wang *et al.*, 2005; Chen *et al.*, 2006; Yu *et al.*, 2008). However, in keratinocytes, the function of P2Y<sub>2</sub>R has been proven to be antimotogenic. Indeed, we previously showed that activation of P2Y<sub>2</sub>R by UTP in keratinocytes alters lamellipodia dynamics, disorganizes actin cytoskeleton in a G $\alpha_q$ -dependent manner, and suppresses serum-induced keratinocyte migration in a wound-healing in vitro assay (Taboubi *et al.*, 2007).

In the present study, we used IGF-I as a highly potent activator of the PI3K-dependent keratinocyte migration, in order to gain insight into the molecular mechanism governing the negative cross-talk between P2Y<sub>2</sub>R and IGF-I/PI3K signaling and its role in the regulation of keratinocyte motility. Our results reveal novel roles of purinergic signals as negative regulators of the IGF-I/PI3K-induced keratinocyte migration. Our work suggests that extracellular nucleotides released in the wound bed may play an important role in the modulation of keratinocyte migration during epidermis reepithelialization after injury.

## MATERIALS AND METHODS

### Reagents and Antibodies

Purinergic agonists and 4-hydroxytamoxifen (4-OHT) were from Sigma Aldrich (Lyon, France). IGF-I was from Amersham Pharmacia Biotech (Les Ulis, France). LY294002, U73122, and pertussis toxin (PTX) were from Calbiochem (Darmstadt, Germany). YM-254890 was a generous gift from J. Takasaki (Astellas Pharma, Ibaraki, Japan; Takasaki *et al.*, 2004). Measurement of [Ca<sup>2+</sup>]<sub>i</sub> rise was performed as previously described (Lee *et al.*, 2001). PIK-75 (N-((1E)-(6-bromoimidazo[1,2-a]pyridin-3-yl)methylene)-N'-methyl-N''-(2-methyl-5-nitrobenzene)sulfonylhydrazide, HCl), a selective inhibitor of p110 $\alpha$  and TGX-221 (( $\pm$ )-7-methyl-2-(morpholin-4-yl)-9-(1-phenylaminoethyl)pyrido[1,2-a]pyrimidin-4-one), a selective inhibitor of p110 $\beta$  were from Calbiochem. Rabbit polyclonal antibodies against (phospho)-Akt (Ser-473), Akt, and (phospho)-GSK-3 $\alpha/\beta$  were purchased from Cell Signaling (Danvers, MA). Rabbit polyclonal anti-G $\alpha_{(q/11)}$ , -IRS-1, -IGF-IR  $\beta$  chain and mouse monoclonal anti-phospho-Tyr (PY) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p85-PI3K antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit antiserum against cortactin was a generous gift of Ellen Van Obberghen-Shilling (Centre Antoine-Lacassagne, Nice, France).

### Cell Lines, Treatments, and Transfections

The human keratinocyte cell line HaCaT (Boukamp *et al.*, 1988), was cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS; Lonza) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Stable HaCaT cell lines were generated by retroviral transfection of HaCaT cells with the vector pLXIN-Myr-p110 $\alpha$ -mER (Myr-p110 $\alpha$ -mER clone) or with the empty vector (vector-transfected clone; Leenders *et al.*, 2004; Pankow *et al.*, 2006). Activation of Myr-p110 $\alpha$ -mER was achieved by addition of 4-OHT (1  $\mu$ M, overnight) to the medium. For inhibition of PI3K, G $\alpha_{(q/11)}$ , PLC $\beta$ , and G $\alpha_{(i/o)}$  proteins, cells were preincubated with either LY294002 (LY; 20  $\mu$ M, 60 min), YM-254890 (YM; 3  $\mu$ M, 5 min), U-73122 (5  $\mu$ M, 30 min), or PTX (100 ng/ml, overnight), respectively. G $\alpha_{(q/11)}$  small interfering RNA (siRNA) nucleofection experiments were performed as previously described (Taboubi *et al.*, 2007). Human endothelial cells (HUVECs) were cultured as previously described (Taboubi *et al.*, 2007).

### Cell Spreading Assays

Cells were preincubated with LY294002 or YM-254890 as indicated above. Wells were precoated with a laminin-5 (LM-5)-enriched matrix as previously described (Taboubi *et al.*, 2007), and then extracellular nucleotides and/or IGF-I were spotted onto the wells just before cell seeding. Cells (1.5  $\times$  10<sup>4</sup> cells/cm<sup>2</sup>) were seeded and incubated for the indicated times at 37°C and then fixed with 3.7% formaldehyde. Three random fields per well from duplicate wells were pictured under a 10 $\times$  objective, and the cell surface of 100 cells per experiment was measured using the ImageJ software (NIH; <http://rsb.info.nih.gov/ij>).

### Migration Assays

Cells were serum-starved overnight, trypsinized, and then seeded on a LM-5-enriched matrix (10<sup>4</sup> cells/cm<sup>2</sup>). Cells were then allowed to migrate at 37°C, 5% CO<sub>2</sub> for 2 h. Migration was analyzed using an inverted Nikon microscope at 20 $\times$  magnification (Melville, NY). Time-lapse recording started after cell adhesion and spreading (20 min). Three fields per well were imaged and followed at 10-min intervals over 2 h with a Coolsnap HQ camera (Photometrics, Tucson, AZ) operated by NIS-elements AR 2.30 software (Nikon). Manual single-cell tracking was performed using Metamorph software (Roper Scientific, Evry, France) as described previously (Sadok *et al.*, 2008). Migration parameters calculated from each individual cell were determined from time-lapse movies. They include total migration distance, distance to origin, velocity, and directional persistence of cell migration. Total migration distance represents the sum of distances between each measurement over a period of 2 h. The distance to origin was determined as the net translocation between the initial and the final position. Velocity was calculated as the total migration distance divided by 2 h. Directional persistence was calculated as the ratio of the distance to origin to the total migration distance. For each parameter, results are expressed as the mean  $\pm$  SD from at least 60 individual cells.

### Immunofluorescence

For cortactin labeling, cells were treated as indicated in the figure legends and then fixed in 3.7% formaldehyde and blocked with 3% bovine serum albumin (BSA). Cells were labeled overnight at 4°C with anti-cortactin serum (1/3000 dilution) and then with the appropriate fluorescent secondary antibody and rhodamine-coupled phalloidin. Cells were observed under immersion oil 63 $\times$ /1.4 Pan Apochromat objectives on a confocal Leica SP5 microscope (Leica Microsystems, Nanterre, France).

### Assays for PI3K Activity

HaCaT cells were cultured to 80% confluence in 10-cm<sup>2</sup> dishes, and then quiescence was induced by incubating cells overnight in serum-free medium containing 0.5% BSA. Cells were then stimulated with IGF-I (50 ng/ml) either alone or with UTP (100  $\mu$ M) for 1, 4, or 10 min at 37°C. Cells were then lysed in 1 ml of Nonidet P-40-containing lysis buffer with protease and phosphatase inhibitors. Equal amounts of protein lysates were incubated with 5  $\mu$ l of anti-p85-PI3K antibody for 1 h at 4°C and then bound with protein A-agarose beads for 1 h at 4°C. Immunoprecipitates were assayed for their content in p110 $\alpha$ -lipid kinase activity by using an inverted competitive ELISA according to the manufacturer's recommendations (Echelon Biosciences, Salt Lake City, UT).

### Immunoblotting and Immunoprecipitation

Keratinocytes were lysed on ice in a buffer containing 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 4 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM PMSF, 1  $\mu$ g/ml leupeptin, and 1  $\mu$ g/ml aprotinin for 15 min at 4°C. Cell lysates were centrifuged for 10 min at 10,000  $\times$  g to eliminate cell debris. Equal amounts of protein (Protein Assay Kit, Bio-Rad, Hercules, CA) were separated by SDS-PAGE and then transferred to Hybond-C nitrocellulose membranes (Amersham Pharmacia Biotech). Membranes were probed with the appropriate primary antibody (2  $\mu$ g/ml) and then with a peroxidase-

conjugated secondary antibody. Bound immunocomplexes were detected using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). For immunoprecipitation, cell lysates were precleared by incubation with 25  $\mu$ l of normal rabbit or mouse IgG serum, followed by incubation with the appropriate primary antibody (5  $\mu$ g/ml) overnight at 4°C. Cell lysates were then incubated with protein A-agarose beads for 1 h at 4°C. The beads were washed three times with the lysis buffer and used for immunoblotting as described above.

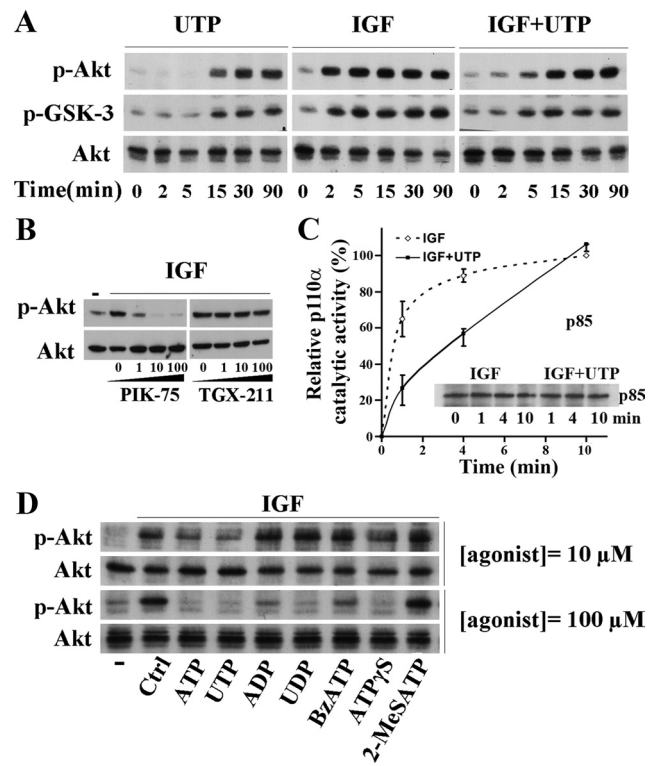
## RESULTS

### UTP Inhibits IGF-I–induced Early Phase Activation of p110 $\alpha$ -PI3K/Akt Pathway

Like other GPCRs, P2Y receptors can activate PI3K pathway (Irinio *et al.*, 2008). Here, we stimulated HaCat cells with 100  $\mu$ M of extracellular UTP, a noncytotoxic concentration widely used to activate P2YRs in keratinocytes (Dixon *et al.*, 1999; Lee *et al.*, 2001; Koizumi *et al.*, 2004; Yoshida *et al.*, 2006; Inoue *et al.*, 2007; Taboubi *et al.*, 2007). Similarly, we found that UTP stimulated the phosphorylation of the ser/thr protein kinase Akt and the glycogen synthase kinase-3 (GSK-3), two PI3K effectors (Figure 1A, left). However, phosphorylation of both proteins was detectable only 15 min after UTP treatment. On the other hand, IGF-I (50 ng/ml) activated the PI3K pathway earlier; within 2 min of treatment (Figure 1A, middle). Noteworthy, costimulation of keratinocytes by IGF-I and UTP resulted in a notable delay in the onset of both Akt and GSK-3 phosphorylation (15 vs. 2 min, Figure 1A, right). Similar results were obtained using ATP instead of UTP (data not shown). Thus, ATP and UTP elicited opposite dual regulatory signals toward PI3K/Akt pathway, i.e., an already reported activating signal and an unusual inhibitory signal revealed when the PI3K pathway was activated by growth factors such as IGF-I.

We previously published that UTP treatment induces the release of a GFP protein fused with the pleckstrin-homology domain of Akt from the plasma membrane indicating a decrease in the amount of plasma membrane PIP<sub>3</sub> (Taboubi *et al.*, 2007). Moreover, we observed that in contrast to wild type Akt, a membrane-targeted mutant of Akt (myr-Akt) was still phosphorylated after UTP cell stimulation (unpublished data). Thus, it is likely that UTP inhibited Akt phosphorylation by disturbing its membrane targeting through the control of PIP<sub>3</sub> amount. As keratinocytes express p110 $\alpha$  and p110 $\beta$  catalytic PI3K subunits (Pankow *et al.*, 2006), we first sought to identify which p110 isoform is responsible for the Akt phosphorylation induced by IGF-I. For this purpose, before IGF-I stimulation, HaCat cells were pretreated with increasing doses of either PIK-75 or TGX-221, two selective pharmacological inhibitors of p110 $\alpha$  and p110 $\beta$  subunit, respectively. Immunoblot analyses shown on Figure 1B revealed that IGF-I failed to stimulate Akt phosphorylation when p110 $\alpha$  was inhibited, whereas no alteration in Akt phosphorylation was observed when p110 $\beta$  was blocked. As a control and in agreement with previous reports that documented activation of p110 $\beta$  by GPCRs (Kurosu *et al.*, 1997; Maier *et al.*, 1999), we found that TGX-221 inhibited the late induction of Akt phosphorylation by UTP (Figure S1A).

Using *in vitro* enzymatic assays of immunopurified PI3K, we showed that IGF-I induced a rise in p110 $\alpha$ -PI3K lipid kinase activity that reached a plateau 2 min after treatment. By contrast, at this time, the assayed lipid kinase function was twofold lower when p110 $\alpha$ -PI3K was purified from cells costimulated with both IGF-I and UTP compared with the one obtained from cells stimulated by IGF-I alone. In the presence of UTP, the amount of produced PIP<sub>3</sub> reached the plateau only 10 min after stimulation (Figure 1C). From these experiments, we concluded that, the loss of Akt phosphorylation

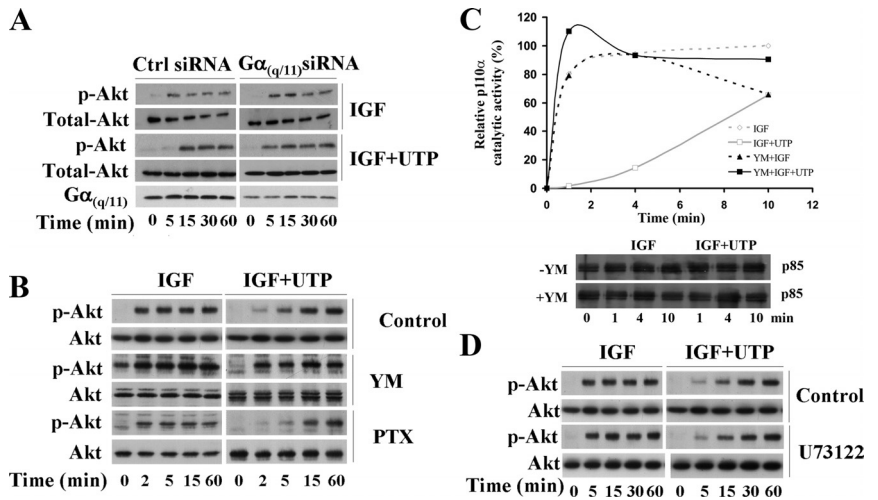


**Figure 1.** UTP inhibits the IGF-I–induced PI3K signaling pathway through P2Y<sub>2</sub>/P2Y<sub>4</sub> receptors. (A) HaCat keratinocytes were stimulated for the indicated times with UTP (100  $\mu$ M), IGF-I (50 ng/ml), or both. (B) Cells were pretreated with increasing doses of p110 $\alpha$  inhibitor (PIK-75) or p110 $\beta$  inhibitor (TGX-211) for 5 min and then stimulated with IGF-I (50 ng/ml, 5 min). (C) Cells were stimulated with IGF-I (IGF; 50 ng/ml) either alone or supplemented with UTP (100  $\mu$ M; IGF+UTP) for 1, 4, and 10 min. Cells were lysed and PI3K was immunoprecipitated using an anti-p85 antibody. p110 $\alpha$  catalytic activity, i.e., production of PIP<sub>3</sub>, was measured in the immunoprecipitated material by inverted ELISA assay. p85 immunoblot shows that equivalent amount of PI3K were analyzed. For more details, see *Materials and Methods*. Data are expressed as a mean  $\pm$  SD from two independent experiments made in triplicates. (D) Cells were stimulated for 5 min with IGF-I (50 ng/ml) either alone (Ctrl) or in the presence of various purinergic receptor agonists at 10  $\mu$ M and 100  $\mu$ M as indicated; (–), untreated cells. Cell lysates were analyzed by Western blot using anti-phospho-Akt (p-Akt), anti-phospho-GSK3 (p-GSK-3), and anti-Akt (Akt) antibodies as indicated. Data shown are representative of three independent experiments.

observed in previous experiments was primarily due to the transient inhibition of IGF-I–induced p110 $\alpha$ -PI3K activation.

Finally, in order to better characterize the purinergic receptor involved in the PI3K pathway inhibition, we carried out pharmacological analyses using a wide range of P2YR agonists. ATP is a broad agonist of P2Y and P2X receptors, whereas UTP is selective for P2Y<sub>2</sub>R and P2Y<sub>4</sub>R. ATP $\gamma$ S can activate P2Y<sub>2</sub>R, but is a more potent agonist of P2Y<sub>11</sub>R; ADP activates P2Y<sub>1</sub>R; 2-MeSATP is selective for P2Y<sub>1</sub>R and P2X<sub>3</sub>R; UDP activates P2Y<sub>6</sub>R, and BzATP is a P2Y<sub>11</sub>R and P2X<sub>7</sub>R agonist (Burnstock, 2006). As shown on Figure 1D, at a concentration of 100  $\mu$ M all agonists (except 2-MeSATP) decreased IGF-I–induced Akt phosphorylation. In contrast, at 10  $\mu$ M, only ATP and UTP (and to a lesser extent ATP $\gamma$ S) were still able to exert this inhibition. As cultured keratinocytes overexpress P2Y<sub>2</sub>R compared with P2Y<sub>4</sub>R (Yoshida *et al.*, 2006) and data not shown), our results strongly suggest

**Figure 2.** UTP inhibits IGF-I-induced PI3K signaling in a  $G_{\alpha_{(q/11)}}$  but not a  $G_{(i/o)}$ -dependent manner. (A) HaCaT keratinocytes were transiently nucleofected with  $G_{\alpha_q}$  siRNA or nontargeting siRNA as a control (Ctrl siRNA) as described in *Materials and Methods*. Forty-eight hours after transfection, cells were stimulated with IGF-I, either alone or in presence of UTP for the indicated times. (B) HaCaT keratinocytes were pretreated with YM-254890 (YM; 3  $\mu$ M, 5 min) or pertussis toxin (PTX; 100 ng/ml, 18 h) to selectively inhibit  $G_{\alpha_{(q/11)}}$  and  $G_{(i/o)}$ , respectively. Cells were then stimulated with IGF-I (50 ng/ml), alone or supplemented with UTP (100  $\mu$ M) for the indicated times. (C) Cells were treated with YM-254890 as in B, and p110 $\alpha$ -PI3K activity was measured as described in Figure 1C. Data are representative of two independent experiments. (D) To inhibit the PLC $\beta$ , cells were pretreated with U73122 (5  $\mu$ M, 30 min) and then treated as in A. Cell lysates were analyzed by Western blot using anti-phospho-Akt (p-Akt) and anti-Akt (Akt) antibodies as indicated. Data shown are representative of three independent experiments.



that P2Y<sub>2</sub>R is presumably responsible for the early and transient inhibition of IGF-I-activated p110 $\alpha$ -PI3K signaling pathway.

#### Extracellular UTP Inhibits IGF-I-induced PI3K Signaling via $G_{\alpha_{(q/11)}}$ in a PLC $\beta$ -independent Manner

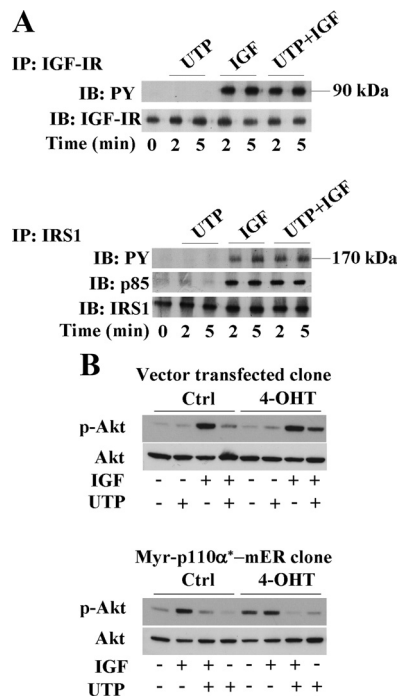
GPCRs can trigger different signals depending on the coupled G protein. P2Y<sub>2</sub>R was initially reported to be mainly coupled to  $G_{\alpha_{(q/11)}}$  (Erb *et al.*, 2006). However, previous studies from the Erb's group have shown that, in addition to  $G_{\alpha_{(q/11)}}$ , P2Y<sub>2</sub>R can also be coupled to and signal through  $G_{\alpha_0}$  or  $G_{\alpha_{12}}$  (Erb *et al.*, 2001; Bagchi *et al.*, 2005; Wang *et al.*, 2005; Liao *et al.*, 2007). To identify the G protein involved in the inhibition of PI3K signaling, we used a validated sequence of siRNA targeting  $G_{\alpha_{(q/11)}}$  (Barnes *et al.*, 2005; Atkinson *et al.*, 2006; Taboubi *et al.*, 2007). In  $G_{\alpha_{(q/11)}}$  siRNA-transfected cells, UTP lost its ability to restrain IGF-I-induced PI3K pathway as evaluated by Akt phosphorylation (Figure 2A). This result was confirmed using YM-254890, a pharmacological cyclic depsipeptide that selectively inhibits  $G_{\alpha_{(q/11)}}$  (Figure 2B; Takasaki *et al.*, 2004). Moreover, YM-254890 blocked the capacity of UTP to inhibit p110 $\alpha$ -PI3K lipid kinase activity (Figure 2C). To test for a possible role of  $G_{(i/o)}$ , we used PTX, a  $G_{(i/o)}$ -specific inhibitor (Albert and Robillard, 2002). PTX treatment of HaCaT cells did not alter the UTP inhibitory effect toward the IGF-I-induced PI3K pathway (Figure 2B, bottom). PTX efficiency was controlled by its ability to inhibit ATP-induced Erk1,2 phosphorylation in human endothelial cells (Figure S1B; Montiel *et al.*, 2006). We further examined the putative role of phospholipase C $\beta$  (PLC $\beta$ ), the main reported downstream effector of  $G_{\alpha_{(q/11)}}$  (Hubbard and Hepler, 2006). For this purpose, we used U73122 (5  $\mu$ M), a potent inhibitor of PLC $\beta$  (Stam *et al.*, 1998). We verified that, in HaCaT cells, at this concentration, U73122 was able to inhibit UTP-induced Erk1,2 phosphorylation (Yang *et al.*, 2004) and intracellular Ca<sup>2+</sup> flux (Lee *et al.*, 2001; Figure S1C) without any cytotoxic effect (not shown). As shown on Figure 2D, addition of U73122 did not alter the ability of UTP to inhibit IGF-I-induced Akt phosphorylation. Taken together, these data indicate that UTP required  $G_{\alpha_{(q/11)}}$ , but not  $G_{(i/o)}$ , to inhibit the p110 $\alpha$ -PI3K pathway. In addition, PLC $\beta$ , although activated by  $G_{\alpha_{(q/11)}}$ , was not involved in this inhibitory pathway.

#### UTP Does Not Interfere with p110 $\alpha$ -PI3K Plasma Membrane Recruitment

We next attempted to specify the molecular mechanism whereby purinergic signaling interfered with PI3K pathway. Neither IGF-IR surface expression (measured by flow cytometry; data not shown), nor IGF-IR autophosphorylation on tyrosine residues (Figure 3A, top) were modified by UTP treatment. Furthermore, UTP did not reduce tyrosine phosphorylation of IRS-1, the immediate downstream substrate of IGF-IR, and did not change the capacity of IRS-1 to recruit the p85-PI3K regulatory subunit (Figure 3A, bottom). Collectively, these results indicate that UTP did not alter assembly of the IGF-IR/IRS-1/PI3K complex. To investigate further, we examined the impact of UTP on the signaling function of an inducible active form of the p110 $\alpha$  catalytic subunit that is targeted to plasma membrane by myristoylation (Myr-p110 $\alpha^*$ -mER; Leenders *et al.*, 2004; Pankow *et al.*, 2006). Activation of Myr-p110 $\alpha^*$ -mER protein was induced by addition of 4-OHT. Figure 3B (top) shows that, in empty vector-transfected cells, 4-OHT had no effect on the basal level of Akt phosphorylation. As expected in these cells, UTP still inhibited IGF-I-induced Akt phosphorylation. In agreement with a previous work (Pankow *et al.*, 2006), we observed that Myr-p110 $\alpha^*$ -mER activation by 4-OHT-induced Akt phosphorylation. Interestingly, this agonist-independent Akt phosphorylation was strongly inhibited by UTP (Figure 3B, bottom). Together, all these results indicate that inhibition of PI3K signaling pathway by UTP occurred downstream of the PI3K recruitment to the plasma membrane.

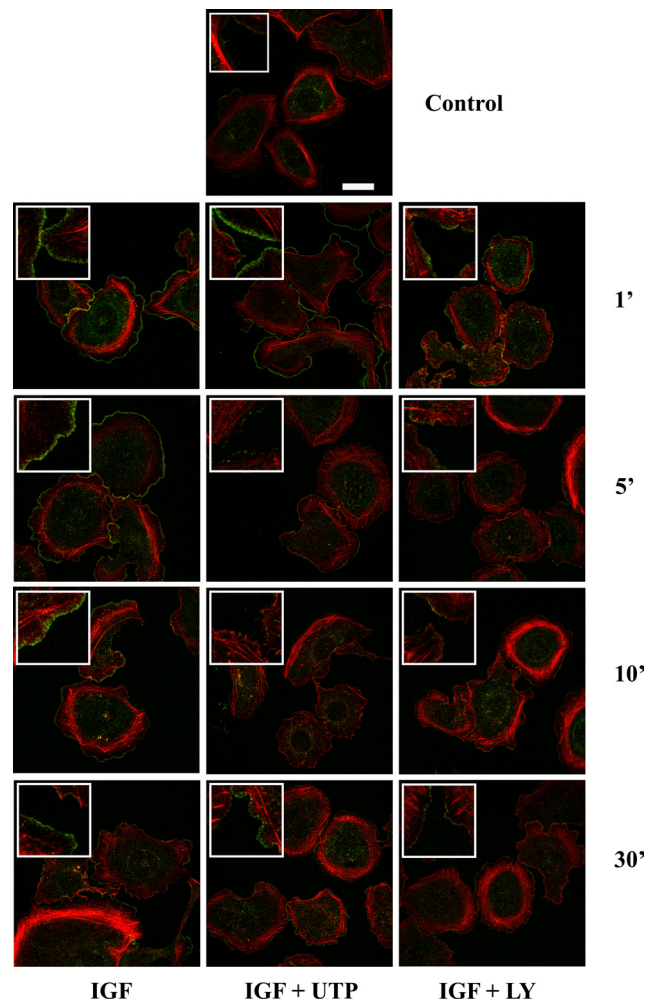
#### Extracellular UTP Blocks PI3K-dependent Cortactin Recruitment to the Lamellipodium

In keratinocytes, lamellipodium extension is driven by p110 $\alpha$ -PI3K activation (Haase *et al.*, 2003; Pankow *et al.*, 2006). In our previous work, we showed that within few minutes UTP treatment induces actin cytoskeleton disorganization and a transient lamellipodium withdrawal (Taboubi *et al.*, 2007). Among the large array of proteins involved in cortical actin regulation, cortactin plays a key role as a promoter of actin filament branching and subsequent lamellipodium extension (Bryce *et al.*, 2005; Kowalski *et al.*, 2005; Ammer and Weed, 2008). Growth factors lead to cortactin



**Figure 3.** UTP inhibits the PI3K signaling pathway downstream of p110 $\alpha$ -PI3K membrane recruitment. (A) HaCaT keratinocytes were stimulated for 2- and 5-min with UTP (100  $\mu$ M), IGF-I (50 ng/ml), or both. Immunoprecipitations were performed on cell lysates with an anti-IGF-I receptor (IGF-IR) antibody (top) or anti-IRS1 antibody (bottom). Immunoprecipitates were analyzed by Western blot using anti-phospho-tyrosine antibody (PY) and anti-p85-PI3K antibody (p85), as indicated. Anti-IRS1 (IRS1) and anti-IGF-IR (IGF-IR) antibodies were used as controls. (B) Myr-p110 $\alpha^*$ -mER-expressing HaCaT clone and vector-transfected clone were treated with either 4-hydroxytamoxifen (4-OHT) or solvent (Ctrl) and then stimulated with IGF-I (50 ng/ml) (IGF) and/or UTP (100  $\mu$ M; UTP) for 5 min. Cell lysates were analyzed by Western blot using anti-phospho-Akt (p-Akt) and anti-Akt antibody (Akt) as loading control. Data shown are representative of three independent experiments.

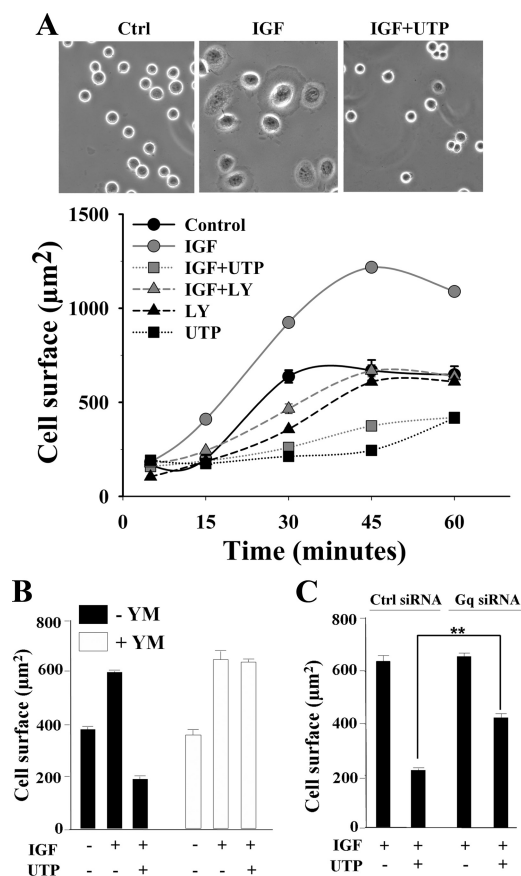
relocalization from internal cytoplasmic region to the cortical actin network. Although the precise mechanism controlling cortactin translocation is not clearly established, it has been reported that, in keratinocytes, PI3K is involved in this process (Ceccarelli *et al.*, 2007). In HaCat cells, we observed a clear cortactin translocation from the cytosol to the rim of membrane ruffles, with a maximal recruitment at early time points of IGF-I stimulation (1 and 5 min) (Figure 4). In LY294002-treated cells, cortactin redistribution was potentially blocked, confirming the involvement of PI3K in this process. As shown in Figure 4, UTP (100  $\mu$ M) transiently perturbed the IGF-I-induced cortactin relocalization. Although we observed a short delay, UTP-induced inhibition of both cortactin recruitment and p110 $\alpha$ -PI3K function were timely correlated (see also Figures 1 and 2). Moreover, the kinetics of the cortactin translocation inhibition induced by UTP is very similar to that of lamellipodium withdrawal described elsewhere (Taboubi *et al.*, 2007), suggesting that cortactin is oppositely regulated by UTP and IGF-I to control lamellipodium formation. Thus, we identified cortactin recruitment to the plasma membrane as a critical early event downstream of the PI3K activation by IGF-I that is inhibited by extracellular UTP and may affect later processes such as cell spreading and motility (see below).



**Figure 4.** UTP blocks PI3K-dependent cortactin membrane translocation. HaCaT keratinocytes were treated or not with LY294002 (LY; 30  $\mu$ M, 60 min) and then stimulated with IGF-I (IGF; 50 ng/ml), either alone or in the presence of UTP (100  $\mu$ M) for the indicated period of time. Untreated cells were used as a control (Ctrl). Before confocal analysis, filamentous actin organization was revealed by phalloidin staining (red), and cortactin was labeled using an anti-cortactin antibody (green). Scale bar, 20  $\mu$ m.

#### Extracellular UTP Inhibits PI3K-induced Keratinocyte Spreading and Motility in a $G_{\alpha(q/11)}$ -dependent Manner

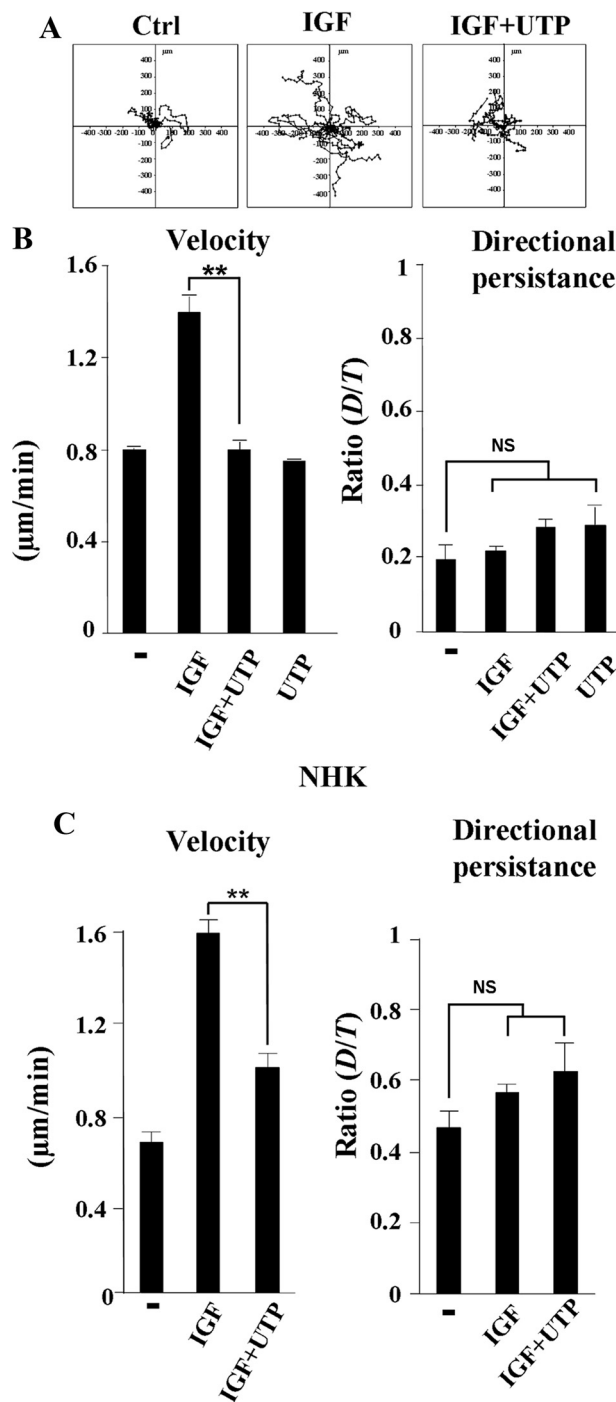
In accordance with data from Watt's group (Haase *et al.*, 2003), 20 min after seeding, IGF-I (50 ng/ml) stimulated lamellipodium formation and increased cell spreading in keratinocytes (Figure 5A, top). In the presence of UTP (100  $\mu$ M), cells remained round and failed to extend any lamellipodium (Figure 5A, top). Quantification of time course cell spreading revealed that after 45 min IGF-I increased by twofold the mean cell size (Figure 5A, bottom). Cells pretreated with LY294002 behave like control cells, indicating that IGF-I-induced cell spreading is largely due to the activation of the PI3K pathway. In presence of UTP (100  $\mu$ M), HaCat cells spread poorly, even 60 min after seeding. Notably, UTP inhibited IGF-I-induced and basal cell spreading, suggesting that P2Y<sub>2</sub>R was not only able to block PI3K-dependent but also PI3K-independent cell spreading. We obtained similar results with normal human keratinocytes (data not shown). As reported above, UTP inhibited PI3K pathway in a  $G_{\alpha(q/11)}$ -dependent manner (see Figure 2).



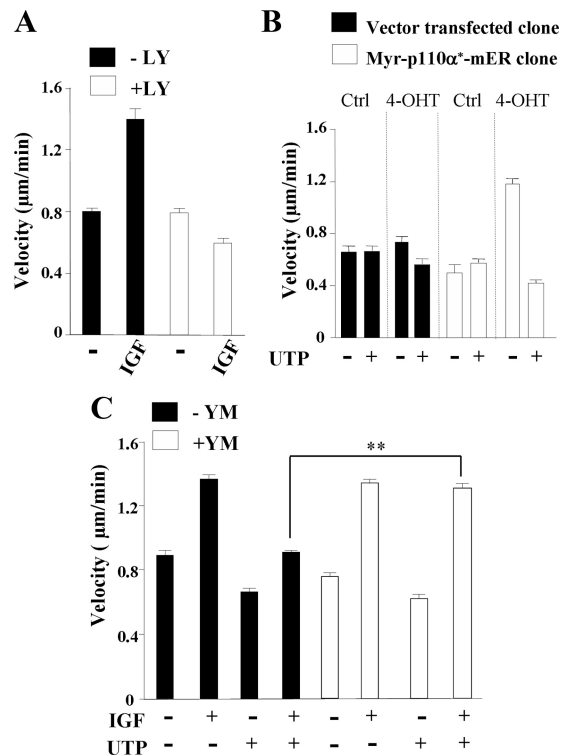
**Figure 5.** UTP inhibits IGF-I-induced PI3K-dependent cell spreading through Gαq activation. (A) HaCaT keratinocytes were allowed to spread on LM-5-enriched matrix in the presence of IGF-I 50 ng/ml (IGF), either alone or supplemented with UTP 100 μM (IGF+UTP). Top, phase-contrast microphotographs show cell-spreading inhibition by UTP 20 min after seeding. Bottom, a time-course spreading of HaCat cells on LM-5. When indicated, cells were preincubated with LY294002 (LY; 30 μM, 60 min). Cell surface was quantified as described in *Materials and Methods*. Data are expressed as the mean ± SEM (n = 100) and are representative of three independent experiments. (B) Cells were pretreated with YM-254890 (YM; 3 μM, 5 min), and then spreading assays were performed as described in A. (C) Cells were nucleofected with a nontargeting siRNA (Ctrl siRNA) or a Gq siRNA. Forty-eight hours after transfection, spreading assays were performed as described in A. Data were statistically analyzed using the Student's *t* test. \*\**p* < 0.001.

Therefore, we next examined whether Gα<sub>(q/11)</sub> activation was also required for UTP to exert its inhibitory effect on PI3K-dependent cell spreading. UTP failed to inhibit IGF-I-induced cell spreading in cells where Gα<sub>(q/11)</sub> function was repressed either by pharmacological inhibition (YM-254890, 3 μM; Figure 5B) or by siRNA-mediated knockdown of its expression (Figure 5C).

Using an *in vitro* wound healing assay, we previously reported that purinergic signaling delays keratinocyte migration (Taboubi *et al.*, 2007). Here, we performed a random two-dimensional motility assay using time-lapse videomicroscopy (Sadok *et al.*, 2008). This technique allows a more precise identification of the migratory parameters that might be affected by purinergic signaling, *i.e.*, cell velocity or directional persistence. Representative migration paths (where the origin of each cell track has been set to the coordinate (x = 0, y = 0) for 10 cells are shown on Figure 6A. Analysis



**Figure 6.** Effect of UTP on IGF-I-induced keratinocyte random migration. (A) Presentation of 10 HaCaT keratinocyte migration paths during 120 min: untreated cells (Ctrl), cells stimulated by IGF-I (50 ng/ml), either alone (IGF) or in combination with UTP (100 μM) (IGF+UTP). (B and C) Motility assays were performed with HaCaT cells (B) or normal human keratinocytes (C; NHK) as described in A. In each experimental condition, trajectory of at least 40 individual cells was analyzed. Cell velocity and directional persistence were calculated from time-lapse movies as described in *Materials and Methods*. Data are expressed as the mean ± SD and are representative of three independent experiments. Data are statistically analyzed using the Student's *t* test. \*\**p* < 0.001; NS, *p* > 0.5.



**Figure 7.**  $G_{\alpha(q/11)}$  activation inhibits p110 $\alpha$ -PI3K-dependent keratinocyte motility. (A) HaCaT keratinocytes were pretreated with or without LY294002 (LY; 10  $\mu$ M) and then with IGF-I (50 ng/ml) and assayed for 2D random migration. (B) Random motility assays were performed with Myr-p110 $\alpha^*$ -mER HaCaT clone ( $\square$ ) and vector-transfected clone ( $\blacksquare$ ). PI3K activation was induced by 4-OHT treatment (4-OHT); solvent-treated cells were used as control (Ctrl). Cells were treated with UTP (100  $\mu$ M) as indicated. (C) HaCaT cells were pretreated with YM-254890 (YM; 3  $\mu$ M, 5 min) and then stimulated with IGF-I (50 ng/ml; IGF) with or without UTP (100  $\mu$ M; UTP) as indicated, and motility assays were performed as described in A. In each experimental condition, trajectories of 40 cells were analyzed, and cell velocity was calculated as described in *Materials and Methods*. Data are expressed as the mean  $\pm$  SD and are representative of three independent experiments. Data were statistically analyzed using the Student's *t* test. \*\**p* < 0.001.

of the trajectory obtained from time-lapse recordings of each individual cell during 2 h, indicated that control untreated and UTP-treated cells migrated slowly ( $0.77 \pm 0.06 \mu\text{m}/\text{min}$ ) whereas IGF-I-stimulated cells exhibited an enhanced migration velocity ( $1.5 \pm 0.1 \mu\text{m}/\text{min}$ ; Figure 6B). Neither IGF-I nor UTP significantly affected cell directionality. Interestingly, in the presence of UTP, IGF-I failed to increase cell velocity ( $0.8 \pm 0.05 \mu\text{m}/\text{min}$ ; Figure 6B). This inhibition was sustained and persisted even 10 h after the beginning of the recording (data not shown). Similar results were obtained when normal human keratinocytes were used instead of HaCaT cells (Figure 6C). Thus, IGF-I did not control cell directionality but promoted keratinocyte migration by increasing cell speed, an essential function which is totally abolished by UTP.

As shown on Figure 7A, PI3K inhibition by LY294002 abrogated the IGF-I-induced increase of cell velocity. To confirm the role of p110 $\alpha$ -PI3K, we conducted a second series of motility assays with vector-transfected or Myr-p110 $\alpha^*$ -mER HaCaT clones. We first verified that these cells behave like untransfected HaCaT cells upon stimulation by IGF-I and UTP (data not shown). As already reported, acti-

vation of Myr-p110 $\alpha^*$ -mER by 4-OHT promotes HaCat migration in wound healing assays (Pankow *et al.*, 2006). Figure 7B shows that, in random motility assays, activation of the p110 $\alpha$ -PI3K mutant by 4-OHT-induced an increase in cell velocity ( $1.2 \pm 0.09 \mu\text{m}/\text{min}$ ,  $\square$ ) compared with empty vector-transfected cells ( $0.47 \pm 0.02 \mu\text{m}/\text{min}$ ,  $\blacksquare$ ). As reported above for IGF-I (see Figure 6), no increase in cell migration persistence was noted (not shown). Importantly, UTP was able to inhibit cell velocity of cells expressing activated Myr-p110 $\alpha^*$ -mER (Figure 7B). Using YM-254890 (3  $\mu$ M), we showed that  $G_{\alpha(q/11)}$  also plays a crucial role in the signaling pathway driven by extracellular UTP to slow down keratinocyte migration (Figure 7C). Taken together, these results clearly show that activation of  $G_{\alpha(q/11)}$  by extracellular nucleotides inhibited p110 $\alpha$ -dependent keratinocyte velocity.

## DISCUSSION

We previously reported that purinergic receptor activation by extracellular nucleotides reduces keratinocyte wound healing by inhibiting growth factor mitogenic effects (Taboubi *et al.*, 2007). An important issue coming from this study was to decipher the molecular mechanism governing this opposite cross-talk between Gq-coupled P2Y receptors and RTKs. Here, we show that P2Y<sub>2</sub>R signaling inhibits IGF-I-induced p110 $\alpha$ -PI3K lipid kinase activity in a  $G_{\alpha(q/11)}$ -dependent manner. Moreover, our data show that extracellular UTP impeded IGF-I-induced PI3K-mediated keratinocyte migration by inhibiting both cell spreading and cell velocity.

As other GPCRs, P2Y<sub>2</sub>R has been reported to mostly transactivate RTKs or to synergistically cross-talk with their downstream effectors (reviewed in Erb *et al.*, 2006). However, this does not constitute an absolute rule. Indeed, several reports clearly show that GPCRs can also dampen the PI3K/Akt pathway, thus inducing physiopathological repercussions, as illustrated in insulin resistance-associated hypertension and several cardiovascular diseases (Velloso *et al.*, 1996; Folli *et al.*, 1997; Howes *et al.*, 2003; Motley *et al.*, 2003). GPCRs can antagonize RTK-induced PI3K/Akt signal transduction using different molecular mechanisms. These include RTK dephosphorylation (Lin *et al.*, 2003), dephosphorylation of the adaptator protein IRS (Ueda *et al.*, 2004), or alteration of IRS-1/p85-PI3K interaction (Folli *et al.*, 1997; Motley *et al.*, 2002, 2003), all events that ultimately can lead to the inhibition of PI3K activation (Velloso *et al.*, 1996; Folli *et al.*, 1997; Ballou *et al.*, 2000; Bousquet *et al.*, 2006). Conversely, GPCRs have also been shown to stimulate phosphatase and tensin homologue on chromosome 10 (PTEN) activation thus impeding PI3K pathway (Sanchez *et al.*, 2005). In HaCat cells, this possibility with regards to P2Y<sub>2</sub>R remains to be investigated in future studies. It has also been shown that GPCR-induced PIP<sub>2</sub> depletion due to PLC $\beta$  activation can compromise PIP<sub>3</sub> production (Howes *et al.*, 2003). However, we report here that in HaCat cells, the U73122-insensitive inhibition of Akt phosphorylation by UTP (Figure 2) indicating that alteration of IGF-I signaling by UTP does not require PLC $\beta$  activation. In parallel with our own observations, other groups have also described PLC $\beta$ -independent inhibition of Akt phosphorylation by GPCR (Ballou *et al.*, 2003; Ueda *et al.*, 2004; Golebiewska and Scarlata, 2008).

Importantly, we show that purinergic signaling reduced IGF-I promoted p110 $\alpha$ -PI3K lipid kinase activity in HaCat cells by more than 60% (Figure 1C). Extracellular nucleotides may therefore trigger intracellular signaling, leading to the inhibition of p110 $\alpha$ -PI3K activation by IGF-I. However, our data supports a model in which UTP does not inhibit the

p110 $\alpha$ -PI3K membrane recruitment, the initial step of its activation by growth factors. Two lines of evidence confirm this. First, UTP-inhibited Akt phosphorylation induced by a wide variety of signals, including cell stimulation by IGF-I (Figures 1–3), epidermal growth factor (EGF; unpublished observation), fetal calf serum (Taboubi *et al.*, 2007). Second, the signaling function of a membrane-targeted active mutant of p110 $\alpha$  subunit (myr-p110 $\alpha^*$ -mER) was also inhibited by UTP (Figure 3). Finally, we did not find any evidence for a dissociation of the IGF-R/IRS-1/p85 complex by UTP (Figure 3).

Using siRNA knockdown approach and YM-254890, a specific pharmacological inhibitor (Uemura *et al.*, 2006), we showed that G $\alpha_{(q/11)}$  is essential to convey the inhibitory signal from P2Y<sub>2</sub>R to PI3K (Figure 2). It has already been reported that Gq-coupled receptors can inhibit PI3K/Akt pathway (Ballou *et al.*, 2000; Motley *et al.*, 2003; Ueda *et al.*, 2004). In particular, *in vitro* and *in vivo* studies from the Lin's group have shown that active G $\alpha_q$  can selectively bind to the p110 $\alpha$  subunit but not to p110 $\beta$  or p110 $\gamma$  and can inhibit its lipid kinase activity (Ballou *et al.*, 2000, 2003, 2006; Bommakanti *et al.*, 2000). However, in HaCat cells, we were unable to coimmunoprecipitate endogenous p110 $\alpha$ -PI3K and G $\alpha_q$ , which does not imply a resilient interaction between these two proteins. G $\alpha_{(q/11)}$  can trigger multiple signals by directly linking various effectors such as PLC $\beta$ , p63RhoGEF, or p110 $\alpha$ -PI3K (Ballou *et al.*, 2003; Rojas *et al.*, 2007). In a recent work, Golebieska and Scarlata (2008) have demonstrated the presence of stable and separate pools of G $\alpha_q$ -PLC $\beta$  and G $\alpha_q$ -PI3K complexes by using Foster resonance energy transfer. Thus, in a given cell type, there may be a different subset of G $\alpha_q$  preassociated with one of these effectors, and these complexes would then selectively direct the signal in either direction, *i.e.*, PLC $\beta$  activation or PI3K inhibition. Finally, because P2Y<sub>2</sub>R are also coupled to other G proteins (Erb *et al.*, 2001; Liao *et al.*, 2007), we cannot completely rule out their possible involvement in PI3K inhibition. However, using PTX we showed that PI3K inhibition did not require the coupling of P2Y<sub>2</sub>R with G $\alpha_{(i/o)}$  (Figure 2). The exact mechanism whereby activation of G $\alpha_q$  by UTP can inhibit p110 $\alpha$ -PI3K function remains unclear and is still under investigation in our laboratory.

In the current study, we consistently observed that UTP inhibited the early phase of the p110 $\alpha$ -PI3K signaling function (1–10 min after cell stimulation by IGF-I). It is now well documented that cell stimulation by RTKs induces two waves of PI3K product accumulation (Jones *et al.*, 1999; Yip *et al.*, 2007). The late peak of PIP<sub>3</sub> (rising between 3 and 7 h) is required for cell cycle progression (Jones *et al.*, 1999), whereas the early and transient PIP<sub>3</sub> accumulation (between 0.5 and 5 min) controls actin remodeling and protrusion formation at the leading edge of migrating cells (Merlot and Firtel, 2003; Mouneimne *et al.*, 2004; Yip *et al.*, 2007; Kolsch *et al.*, 2008). In agreement with these reports, we observed a fast translocation of the actin-nucleating protein cortactin to the cell edges in IGF-I stimulated HaCat cells (Figure 4). Moreover, this early PI3K-dependent cortactin relocalization was inhibited by UTP in a time correlated manner with the inhibition of PI3K by Gq-coupled P2Y<sub>2</sub>R (Figures 1, 2 and 4). It has also been proposed that the early PI3K activation can alter the conformation of other signaling proteins, *e.g.*, the protein kinase C and therefore can trigger signals over a relatively long period of time (Balciunaite and Kazlauskas, 2001). Thus, it is actually conceivable that UTP, by altering the first wave of PI3K activation, may have an immediate profound impact on actin dynamics and may later reduce cell spreading and cell motility (Figures 5 and 7).

Finally, it should be noted that in sharp contrast to the data presented here and our own previously published results, several other publications have reported that P2Y<sub>2</sub>R stimulates cell migration in several other cell types (Chaulet *et al.*, 2001; Pillois *et al.*, 2002; Bagchi *et al.*, 2005; Kaczmarek *et al.*, 2005; Wang *et al.*, 2005; Chen *et al.*, 2006; Yu *et al.*, 2008). Studies from the Erb's group have partially deciphered the signaling pathway downstream of P2Y<sub>2</sub>R that promotes astrocyte migration. The authors reported that P2Y<sub>2</sub>R interacts with  $\alpha_v$  integrin to access and activate G<sub>0</sub> or G<sub>12</sub> and in turn induce cell migration through the activation of downstream pathways (Erb *et al.*, 2001; Bagchi *et al.*, 2005; Liao *et al.*, 2007). Interestingly, they provide several data indicating that a mutant of P2Y<sub>2</sub>R that is unable to bind  $\alpha_v$  integrin and to promote cell migration still activates Gq/PLC $\beta$  pathway. Thus, in a given cell type, it appears that P2Y<sub>2</sub>R may be coupled to different G proteins and may therefore be able to induce distinct signaling pathways.

Acute wounds normally heal by a well-orchestrated, tightly regulated series of processes including hemostasis, inflammation, and tissues formation and remodeling. In aging individuals or in a pathophysiological context such as diabetes mellitus or venous insufficiency, skin lesions most often result in chronic nonhealing ulcers (Menke *et al.*, 2007). Exogenous growth factors have been reported as important mediators that enhance tissues remodeling and induce faster wound closure (Werner and Grose, 2003). During the inflammatory phase of skin wound healing, extracellular nucleotides are abundant and most likely participate to the regulation of numerous events such as platelet activation (Gachet, 2008), pain transmission (Dussor *et al.*, 2009), or neutrophil and phagocyte recruitment (Chen *et al.*, 2006; Elliott *et al.*, 2009). Our current finding that P2Y<sub>2</sub>R inhibits signaling of mitogenic growth factors on cultured keratinocytes suggests that extracellular nucleotides may have additional functions during wound healing by delaying the reepithelialization phase. Thus, an excess of extracellular ATP in chronic wound might be deleterious. Expanding our understanding of this pathway in wound healing, maybe then translated to clinical benefit in the form of preventive or curative treatments of wound healing disorders.

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