The P2Y₂ Nucleotide Receptor Interacts with α₅ Integrins to Activate Gₒ and Induce Cell Migration

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Extracellular ATP and UTP induce chemotaxis, or directed cell migration, by stimulating the G protein-coupled P2Y₂ nucleotide receptor (P2Y₂R). Previously, we found that an arginine-glycine-aspartic acid (RGD) integrin binding domain in the P2Y₂R enables this receptor to interact selectively with α₁β₁ and α₅β₃ integrins, an interaction that is prevented by mutation of the RGD sequence to arginine-glycine-glutamic acid (RGE) (Erb, L., Liu, J., Ockerhausen, J., Kong, Q., Garrad, R. C., Griffin, K., Neal, C., Krugh, B., Santiago-Perez, L. I., Gonzalez, F. A., Gresham, H. D., Turner, J. T., and Weisman, G. A. (2001) J. Cell Biol. 153, 491–501). This RGD domain was found to be necessary for coupling the P2Y₂R to Gₒ, but not Gₛ-mediated intracellular calcium mobilization, leading us to investigate the role of P2Y₂R interaction with integrins in nucleotide-induced chemotaxis. Here we show that mutation of the RGD sequence to RGE in the human P2Y₂R expressed in 1321N1 astrocytoma cells completely prevented UTP-induced chemotaxis as well as activation of Gₒ, Rac, and Vav2, a guanine nucleotide exchange factor for Rac. UTP also increased expression of vitronectin, an extracellular matrix protein that is a ligand for α₁β₁/β₃ integrins, in cells expressing the wild-type but not the RGE mutant P2Y₂R. P2Y₂R-mediated chemotaxis, Rac and Vav2 activation, and vitronectin up-regulation were inhibited by pretreatment of the cells with anti-α₁β₁ integrin antibodies, anti-α₁ integrin antisense oligonucleotides, or the Gₒ inhibitor, pertussis toxin. Thus, the RGD-dependent interaction between the P2Y₂R and α₅ integrins is necessary for the P2Y₂R to activate Gₒ and to initiate Gₒ-mediated signaling events leading to chemotaxis.

Chemotaxis is the movement of a cell in response to a chemical gradient and is required for many physiological events, including embryonic development, immune system function, and wound healing (2–4). The process of chemotaxis is also important for understanding diseases that result from aberrant cell migration, such as chronic inflammation, atherosclerosis, and cancer metastasis. To migrate, cells must establish dynamic and highly regulated adhesive interactions with the extracellular matrix, which are mediated by integrin receptors (3). For example, recent studies have shown that α₅ integrins play an important role in controlling cell adhesion, spreading, and motility in several cell types, including human vascular smooth muscle cells and pancreatic beta cells (5, 6). Upon activation, many types of integrin receptors cluster together and recruit a host of cytoskeletal and cytoplasmic proteins into specialized adhesive structures called focal adhesions. These focal adhesion complexes not only serve as a physical link between the extracellular and intracellular matrix but also are important sites of signal transduction for integrins and many other types of receptors that mediate cell migration (7). Chemotaxis also requires a cell to assume a polarized morphology that is controlled by cell surface receptors that activate the Rho family of GTPases, including Cdc42, Rac, and Rho (8, 9). Upon activation of a chemoattractant receptor, Cdc42 and Rac localize at the leading edge of a cell and control directional movement and the formation of lamellipodia containing highly branched actin filaments, respectively (8). Rho localizes at the rear and sides of a cell and controls the formation of contractile actin-myosin stress fibers (10). Together, these GTPases promote cell migration toward a chemoattractant by mediating extension of the actin cytoskeleton at the front edge of the cell and retraction of the cytoskeleton at the rear edge of the cell.

Recent studies have shown that G protein-coupled receptors (GPCRs) regulate Rac and Rac-dependent lamellipodia formation by activating the G₁α family of heterotrimeric G proteins, whereas activation of Rho and Rho-dependent stress fiber formation are controlled by the G₁₂/₁₃ family (10). Furthermore, studies have shown that the β₄ subunits of G₁α are responsible for activation of Rac guanine nucleotide exchange factors (RacGEFs) that, in turn, activate Rac, whereas the α₂ subunits of G₁₂/₁₃ are responsible for activation of RhoGEFs (8, 11).

The P2Y₂ nucleotide receptor (P2Y₂R), a GPCR activated by extracellular ATP or UTP, is an important regulator of monocyte chemotaxis (12). Although the P2Y₂R is normally expressed in monocytes, neutrophils, and other immune cells (13), recent studies have shown that the P2Y₂R is up-regulated in epithelial and vascular tissue in response to injury or stress (14). Upon activation, the P2Y₂R mediates a variety of biological functions, including mitogenesis, angiogenesis, vasodilatation, chemotaxis, and inflammation (15, 16). Furthermore, recent work from our group has demonstrated that arterial stress in rabbit carotid arteries induces up-regulation of the P2Y₂R in vascular endothelium and smooth muscle and that in vivo activation of the P2Y₂R by UTP leads to a 7-fold increase in intimal thickening and a 4-fold increase in leukocyte infiltration into the neointima (12). Thus, the P2Y₂R represents an exciting target for wound healing as well as chronic inflammatory diseases.

Previously, we reported that the P2Y₂R contains a consensus RGD integrin binding domain in the first extracellular loop that interacts with...
\( \alpha_\beta_1 \) and \( \alpha_\beta_5 \) integrins (1). Furthermore, we found that the RGD domain in the P2Y_{R} is required for G_{i}- but not G_{q}-mediated calcium signaling (1), leading us to speculate that \( \alpha_\beta \) integrin interaction with the P2Y_{R} is important for nucleotide-induced chemotaxis. The purpose of the present study was to evaluate the role of \( \alpha_\beta \) integrin interaction with the P2Y_{R} in nucleotide-induced chemotaxis and to identify upstream signaling events involved in P2Y_{R}-mediated chemotaxis.

**MATERIALS AND METHODS**

**Cell Culture and Transfection**—Human 1321N1 astrocytoma cells were stably transfected with cDNA encoding a hemagglutinin-tagged wild-type (WT) or RGE mutant P2Y_{R} using the pLXSN retroviral vector, as described previously (17). The cells were cultured in DMEM containing 5% fetal bovine serum, 100 units/ml penicillin, 100 \( \mu \)g/ml streptomycin, and 500 \( \mu \)g/ml Geneticin (G418, Invitrogen) at 37 °C in a humidified atmosphere of 5% CO\(_2\) and 95% air. Cell surface expression of the hemagglutinin-tagged WT or RGE mutant P2Y_{R} was determined by flow cytometry, as described previously (18). Surface expression of endogenous \( \alpha_\beta \) integrin in 1321N1 cells expressing the WT or RGE mutant P2Y_{R} was quantitated by flow cytometry using a 1:100 dilution of mouse monoclonal anti-human \( \alpha_\beta \) antibody (P2W7, Santa Cruz Biotechnology) as the primary antibody and a 1:100 dilution of goat anti-mouse IgG-fluorescein isothiocyanate (Santa Cruz Biotechnology) as the secondary antibody. Cells were washed with phosphate-buffered saline, fixed, and analyzed on an EPICS 753 flow cytometer (Coulter Corp., Hialeah, FL). Human 1321N1 cells expressing WT P2Y_{R} were transiently transfected with 5 \( \mu \)g of sense or antisense S oligonucleotides for \( \alpha_\beta \) integrins, dominant negative (DN) Rac1 cDNA (T7N, Upstate Biotechnology) in pUSEamp (+), or DN Vav2 (L342R/L343S, a double mutant with an inactive dbl domain) in pCMV5 (19) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. DN Vav2 was generously provided by Dr. Paola A. Marignani (Dalhousie Medical Research Foundation). The transiently transfected cells were cultured for an additional 24 h in DMEM supplemented with 5% fetal bovine serum, suspended by trypsinization, washed, resuspended in serum-free DMEM, and used in migration assays. Transiently transfected cells used for Vav2 or Rac activation assays were serum-starved for 24 h before UTP treatment.

**Migration Assay**—Cell migration assays were performed with 3-\( \mu \)m pore size Transwells (Costar) as described (15). In brief, cells were suspended by trypsinization, washed, and resuspended in 100 \( \mu \)l of serum-free DMEM (5 \( \times \) 10\(^4\) cells) and placed in the upper chamber of the Transwells. The lower chamber was filled with 500 \( \mu \)l of serum-free DMEM supplemented with varying concentrations of UTP (Amersham Biosciences), as indicated. The cells were allowed to migrate for 16 h at 37 °C. Cells migrating to the lower side of the polycarbonate membrane were fixed with cold methanol and stained with Accustain (Sigma). Cells were counted in 10 microscopic fields at 20\(^\times\) magnification. Analysis was performed on three Transwells for each condition, and each experiment was repeated three times. For antibody (Ab) inhibition studies, rabbit anti-human vitronectin (Vitronectin 65), mouse anti-human \( \alpha_\beta \) integrin (P2W7), mouse anti-human \( \alpha_\beta_5 \) (P1F 76) (Santa Cruz Biotechnology), and control anti-IgG1 Abs (Sigma) were added to both chambers at the same time as the nucleotide.

Chemokinetic assays were performed with the Cell Motility Hit kit (Cellomics, Pittsburgh, PA) following the manufacturer’s instructions. Cell suspensions (500 cells) were added to each well of a collagen-coated 96-well microplate containing a lawn of microscopic blue fluorescent beads. After 16 h of incubation at 37 °C with the indicated reagents, the cells were washed and fixed, and phagokinetic tracks were visualized under a phase contrast microscope at 10\(^\times\) magnification. The images were captured on a digital camera using Northern Eclipse software (Empix Imaging Inc., Mississauga, Ontario, Canada).

**Immunoblot Analysis**—Immunoblot analysis was done as described previously (1). Vitronectin expression and Akt phosphorylation were detected by immunoblotting with a 1:1000 dilution of rabbit anti-human vitronectin (Santa Cruz Biotechnology) and 1:1000 dilution of rabbit anti-human phospho-Akt (Ser-473), respectively (Cell Signaling Technology), as the primary Abs and a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) as the secondary Ab. For signal normalization membranes were probed with 1:1000 dilution of rabbit anti-human actin Ab (Cytoskeleton Inc.).

**Rac and Vav2 Phosphorylation**—Human 1321N1 cells transfected with the WT or RGE mutant P2Y_{R} were plated on uncoated 100-mm tissue culture dishes, serum-starved for 24 h, and stimulated with UTP for 5 min. Rac-GTP was precipitated from the cells with Rac/Cdc42 assay reagent (PAK-1 PBD-agarose) comprised of the P-21 binding domain of human PAK-1 bound to glutathione-agarose (Upstate Biotechnology) following the manufacturer’s instructions. SDS-PAGE was performed with the precipitated protein. Rac activity was detected by immunoblotting with a 1:1000 dilution of mouse anti-human Rac (clone 23A8, Upstate Biotechnology) as the primary Ab and a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG as the secondary Ab (Santa Cruz Biotechnology).

Vav2 phosphorylation was analyzed by immunoprecipitation of Vav2 with 2 \( \mu \)g of mouse anti-human phosphotyrosine Ab (Upstate Biotechnology) followed by immunoblotting of the precipitated protein with a 1:500 dilution of rabbit anti-human Vav2 (Zymed Laboratories Inc.) as the primary Ab and a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) as the secondary Ab. For normalization of the signal, aliquots of total cell lysates were subjected to SDS-PAGE and immunoblotted with 1:1000 dilution of mouse anti-human Rac or rabbit anti-human Vav2 as the primary Abs, which bind to Rac or Vav2, respectively, independent of their phosphorylation state.

\( \alpha_\beta \) Antisense S Oligonucleotides—Sequences of phosphorothioate-derivatized oligonucleotides including the translation initiation site were as follows: \( \alpha_\beta \) sense, 5'-GCCTTCTTCCTGCTAGCGCTTTC-3'; \( \alpha_\beta \) antisense, 5'-CGGAAGGCGCTACGCCAAAAG-3'. The sequences were checked for uniqueness using the National Center for Biotechnology Information’s Local Alignment Search Tool (BLAST). WT P2Y_{R}-transfected 1321N1 cells were transiently transfected with \( \alpha_\beta \) sense and antisense S oligonucleotides as described above. Transfected cells were harvested after 24 h, and total cell lysates were analyzed by immunoblot analysis to evaluate the efficiency of \( \alpha_\beta \) suppression by antisense oligonucleotide treatment. Transfected cells used for detection of Vav2 and Rac phosphorylation or vitronectin expression were serum-starved for an additional 24 h and treated with UTP for 5 min or 8 h, respectively.

\([^{35}S]\)GTP\(_{\gamma}\)S Binding Assay—Membranes (40 \( \mu \)g of protein) were isolated from 1321N1 cells transfected with the WT or RGE mutant P2Y_{R}, as described previously (20), and incubated in assay buffer (50 mM Tris-Cl, pH 7.4, 100 mM NaCl, 5 mM MgCl\(_2\), 1 mM EDTA, 1 mM dithiothreitol, 1 \( \mu \)M guanosine 5'-diphosphate) with 1X protease inhibitor mixture (Roche Applied Science) and 50 nCi of \([^{35}S]\)GTP\(_{\gamma}\)S (1250 Ci/mmol, PerkinElmer Life Sciences) containing the indicated concentration of UTP. Membranes were incubated for 15 min at 30 °C, and the binding assay was terminated by the addition of 0.5 ml of ice-cold buffer containing 50 mM Tris-Cl, pH 7.4, 100 mM NaCl, and 5 mM MgCl\(_2\). The samples were centrifuged at 45,000 rpm for 15 min at 4 °C, and the resulting pellets were resuspended in 500 \( \mu \)l of solubilization buffer (100 mM Tris, pH...
7.4, 200 mM NaCl, 1 mM EDTA, 1.25% (v/v) Nonidet P-40, 0.2% (w/v) SDS, and 1/100 protease inhibitor mixture. Extracts were incubated for 16 h at 4 °C with mouse anti-human Gαo (Sigma) or rabbit anti-human Gαq/11 (Santa Cruz Biotechnology) Abs in the presence of 50 μl of a 50% protein G-agarose suspension (Upstate Biotechnology). The immune complexes were sedimented by centrifugation at 10,000 rpm for 10 min in a microcentrifuge and washed 3 times in wash buffer (50 mM Tris-Cl, pH 7.4, 100 mM NaCl, and 5 mM MgCl₂). [35S]GTPγS binding in the immunoprecipitates was quantified by liquid scintillation spectrometry. The presence of Gαo and Gαq/11 subunits in the immunoprecipitates was confirmed by immunoblotting with 1:1000 dilution of rabbit anti-human Gαo (Calbiochem) and rabbit anti-human Gαq/11 (Santa Cruz Biotechnology) as the primary Abs and a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG as the secondary Ab.

RESULTS
An RGD Integrin Binding Domain in the P2Y₂R Is Required for UTP-induced Cell Migration—We previously reported that the interaction between the P2Y₂R and αᵢ integrins is prevented by mutation of the P2Y₂R RGD sequence to RGE (1). To determine whether the RGD integrin binding domain in the P2Y₂R is important for P2Y₂R-mediated chemotaxis, a modified Boyden chamber assay was used. The results showed that UTP induced a concentration-dependent increase in the migration of cells transfected with the WT P2Y₂R in contrast to cells transfected with the RGE mutant P2Y₂R or vector alone (Fig. 1A). The initial migratory response to UTP in P2Y₂R-transfected cells was observed 8 h after UTP treatment, whereas the maximal response was observed after 16 h. Also, we found that P2Y₂R-transfected cells did not migrate significantly when UTP was placed in the upper chamber instead of the lower chamber or when equal concentrations of UTP were placed in both chambers (data not shown), indicating that UTP acts as a chemoattractant in cells expressing the WT P2Y₂R. To demonstrate that the RGD integrin binding domain of the P2Y₂R is essential for chemokinesis, 1321N1 cells transfected with the WT or RGE mutant P2Y₂Rs were plated on a lawn of microscopic fluorescent beads in collagen-coated 96-well plates. UTP caused cells expressing the WT P2Y₂R to migrate and phagocytose the beads leaving behind phagokinetic tracks, whereas UTP-induced migration of cells expressing the RGE mutant P2Y₂R or the pLXSN vector was significantly restricted (Fig. 1B). The cell transfectants expressed similar surface levels of WT and RGE mutant P2Y₂Rs and αᵢ integrins, suggesting that variations in the magnitude of UTP-induced migration were not due to differences in cell-surface protein levels (Fig. 1C). Thus, the RGD integrin binding domain in the P2Y₂R is required for UTP-induced migration of 1321N1 cells.
domain of the P2Y<sub>2</sub>R is apparently required for UTP-induced chemotaxis and chemokinesis.

Role of Vitronectin in P2Y<sub>2</sub>R-mediated Cell Migration—Vitronectin is a glycoprotein that is found in plasma, platelets, and extracellular matrices. Several lines of evidence suggest that vitronectin plays an important role in vascular smooth muscle cell migration (21, 22). Extracellular matrix proteins, such as vitronectin and osteopontin, are RGD-containing ligands for α<sub>v</sub>β<sub>3</sub/>β<sub>5</sub> integrins and act as chemotactic factors for cells expressing these integrins (15). Because UTP can induce expression of osteopontin via activation of the P2Y<sub>2</sub>R (12), we speculated that variations in the expression levels of extracellular matrix proteins may contribute to the different rates of UTP-induced cell migration for cells expressing the WT and RGE mutant P2Y<sub>2</sub>R. We found that UTP induced a dose-dependent increase in levels of vitronectin (Fig. 2), suggesting that the P2Y<sub>2</sub>R requires interaction with α<sub>v</sub> integrins to activate Rac and Vav2. Furthermore, transient transfection of 1321N1 cells expressing the WT P2Y<sub>2</sub>R with a dominant negative mutant of Rac (Rac DN) and Rac G-TPase activity by Rac DN transfection inhibited UTP-induced Rac1 activation (Fig. 3B), consistent with the role of Rac2-dependent Rac1 activation in lamellipodia formation and cell spreading in other cell lines (19).

P2Y<sub>2</sub>R-mediated Cell Migration Is Dependent on Rac GTPase Activity—To verify that Rac GTPase activity is necessary for P2Y<sub>2</sub>R-mediated cell migration, 1321N1 cells expressing the WT P2Y<sub>2</sub>R were transiently transfected with a dominant negative mutant of Rac (Rac DN). Rac DN-transfected cells were seeded into the upper chamber of Transwells, and 100 μM UTP was added to the lower chamber as indicated. Cell migration was evaluated 16 h after UTP stimulation. The data shown are representative of results from three experiments performed in triplicate where the asterisk (*, p < 0.05) indicates a significant difference from UTP-stimulated control (C).

Expression and Functional Activity of α<sub>v</sub> Integrins Is Essential for UTP-induced Cell Migration, Vav2 and Rac Activation, and Vitronectin Up-regulation—To determine whether α<sub>v</sub> integrins are involved in P2Y<sub>2</sub>R-mediated chemotaxis, we used antisense S oligonucleotide to inhibit expression of α<sub>v</sub>. Transfection of 1321N1 cells expressing the WT P2Y<sub>2</sub>R with antisense oligonucleotide significantly suppressed α<sub>v</sub> expression (Fig. 4A) and completely inhibited UTP-induced cell migration (Fig. 4B). Similarly, transfection of 1321N1 cells expressing the WT P2Y<sub>2</sub>R with antisense oligonucleotide inhibited UTP-induced Vav2 and Rac activation by ~50% and ~60%, respectively, and vitronectin expression by ~75% (Fig. 4C). The α<sub>v</sub> sense oligonucleotide did not significantly inhibit UTP-induced cell migration, Vav2 and Rac activation, or vitronectin up-regulation (Figs. 4, B and C).

To further evaluate the role of α<sub>v</sub> integrins in P2Y<sub>2</sub>R-mediated cell migration, we tested the effect of anti-integrin Abs on UTP-induced
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Involvement of $G_o$ Signaling in P2Y$_2$R-mediated Cell Migration—Per-tussis toxin (PTX), which inhibits $G_{13}$, signaling, has been shown to block chemotaxis mediated by chemokine receptors (29). Previous work from our laboratory has shown that PTX partially inhibits intracellular Ca$^{2+}$ mobilization mediated by the WT P2Y$_2$R but does not block Ca$^{2+}$ mobilization mediated by the RGE mutant P2Y$_2$R, suggesting that the RGD integrin binding domain is required for the P2Y$_2$R to activate $G_o$ (1). Here, we directly investigated whether the RGD domain in the P2Y$_2$R is required for activation of $G_o$. Accordingly, membrane extracts were prepared from 1321N1 cells expressing the WT or RGE mutant P2Y$_2$R and stimulated with 1 mM UTP in the presence of [35S]GTP. Immunoprecipitation of G$\alpha_o$ or G$\alpha_q$ with anti-G$\alpha_o$ or anti-G$\alpha_q/11$ Abs indicated that UTP induced a 2-fold increase in [35S]GTPyS binding to G$\alpha_o$ or G$\alpha_q$ in cells expressing the WT P2Y$_2$R, whereas no increase in [35S]GTPyS binding to G$\alpha_q$ was seen in cells expressing the RGE mutant integrin binding domain in the P2Y$_2$R is required for activation of G$\alpha_q$, but not G$\alpha_o$ and suggest that integrin interaction with the P2Y$_2$R is important for access to specific heterotrimeric G proteins involved in cell migration. Furthermore, we found that PTX inhibited UTP-induced cell migration by 70–80% in 1321N1 cells expressing the WT P2Y$_2$R (Fig. 7A), indicating that G$\alpha_o$ activation is involved in P2Y$_2$R-mediated cell migration. PTX also completely inhibited UTP-induced Vav2 and Rac activation and vitronectin up-regulation (Fig. 8B) in 1321N1 cells expressing the WT P2Y$_2$R, indicating that all of these responses are dependent on G$\alpha_o$ activity.

P2Y$_2$R-mediated Akt Phosphorylation Does Not Require Integrin Interaction—The phosphatidylinositol 3-kinase/Akt pathway is known to regulate actin reorganization and cell migration by some, but not all, chemokine receptors (30, 31), and a recent study has shown that extra-cellular ATP and UTP can increase migration of human umbilical vein endothelial cells in a phosphatidylinositol 3-kinase-dependent manner (32). Therefore, we investigated whether the phosphatidylinositol 3-kinase/Akt pathway is involved in chemotaxis mediated by the P2Y$_2$R expressed in 1321N1 cells. We found that the phosphatidylinositol 3-kinase inhibitor LY294002 inhibited Akt phosphorylation induced by UTP but failed to significantly inhibit UTP-induced migration of 1321N1 cells expressing the WT P2Y$_2$R (Supplemental Fig. S1). We also found that both the WT and RGE mutant P2Y$_2$R could activate Akt, but the RGE mutant P2Y$_2$R required ~1000-fold higher concentrations of phosphatidylinositol 3-kinase/Akt pathway is involved in chemotaxis mediated by the P2Y$_2$R expressed in 1321N1 cells. We found that the phosphatidylinositol 3-kinase inhibitor LY294002 inhibited Akt phosphorylation induced by UTP but failed to significantly inhibit UTP-induced migration of 1321N1 cells expressing the WT P2Y$_2$R (Supplemental Fig. S1). We also found that both the WT and RGE mutant P2Y$_2$R could activate Akt, but the RGE mutant P2Y$_2$R required ~1000-fold higher concentrations of

migration and upstream signaling events in 1321N1 cells transfected with the WT P2Y$_2$R. The increase in migration of P2Y$_2$R-transfected cells in response to UTP was inhibited by anti-$\alpha_v$ and anti-$\alpha_\beta_3$ Abs in a dose-dependent manner, whereas negative control Ab (anti-IgG) did not inhibit UTP-induced cell migration (Fig. 5A). UTP-induced chemokinesis of P2Y$_2$R-transfected cells was also inhibited by anti-$\alpha_v$-$\beta_3$ Ab at a concentration of 50 $\mu$g/ml (Fig. 5B), indicating that $\alpha_v$ integrins are required for UTP-induced cell migration. Likewise, Rac and Vav2 activation and vitronectin up-regulation in response to UTP were significantly inhibited by treatment with anti-$\alpha_v$-$\beta_3$ Ab but not with Ab against another integrin subtype ($\alpha_\sigma$) that is also expressed in 1321N1 cells (Fig. 6) (note: fluorescence-activated cell sorter analysis indicated that $\alpha_\beta_3$ and $\alpha_v$ integrins, but not $\alpha_\beta_3$, are expressed in 1321N1 cells (data not shown)).

Expression of $\alpha_v$ integrin is required for P2Y$_2$R-mediated cell migration, Vav2 and Rac activation, and vitronectin up-regulation. Human 1321N1 cells expressing the WT P2Y$_2$R were transfected with 5 $\mu$g of $\alpha_v$ antisense (AS) or sense (S) oligonucleotides, as described under “Materials and Methods”. A, expression of $\alpha_v$ integrin in these cells was determined by immunoblot analysis. B, transfected cells were seeded into the upper chamber of Transwells, and 100 $\mu$m UTP was added to the lower chamber, as indicated. Cell migration was evaluated 16 h after UTP stimulation. C, transfected cells were treated with 100 $\mu$m UTP for 5 min (to measure Vav2 and Rac activity) or 8 h (to measure vitronectin up-regulation) as indicated. The data shown are representative of results from three experiments (A and C) or the means ± S.E. of results from three experiments performed in triplicate, where the asterisk (*, $p < 0.05$) indicates a significant difference from UTP-stimulated control (8).

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UTP as compared with the WT P2Y2R (Fig. 9A), which is similar to the agonist potency of the RGE mutant P2Y2R in the stimulation of intracellular Ca2+ mobilization, extracellular signal-regulated kinase 1/2 phosphorylation (1), and inositol phosphate accumulation (33). Thus, at high UTP concentrations both the WT and the RGE mutant P2Y2Rs are equally efficacious in the activation of Akt, extracellular signal-regulated kinase 1/2, intracellular Ca2+ mobilization, and inositol phosphate formation. And, although untested, it was recently suggested that the RGE mutation in the P2Y2R may affect these pathways by decreasing the agonist binding affinity of the P2Y2R (33). We next used PTX to evaluate the role of Go in P2Y2R-mediated Akt phosphorylation. PTX caused a 50% inhibition of Akt phosphorylation induced by UTP in 1321N1 cells expressing the WT P2Y2R, whereas Akt phosphorylation was insensitive to PTX treatment in cells expressing the RGE mutant receptor (Fig. 9B). Therefore, we conclude that P2Y2R-mediated Akt phosphorylation in 1321N1 cells occurs by both Gα- and Gq-dependent pathways, although UTP-induced cell migration is largely Gq-dependent. Moreover, the involvement of the phosphatidylinositol 3-kinase/Akt pathway in P2Y2R-mediated migration appears to be cell type-specific and does not play a significant role in P2Y2R-mediated chemotaxis in 1321N1 cells.

DISCUSSION

Previously we found that an integrin binding sequence (RGD) in the G protein-coupled P2Y2R promotes interaction with αβ3/β3 integrins and is required for nucleotide-induced activation of Gαq but not Gαi-mediated intracellular calcium mobilization (1). In the present study we have shown that the P2Y2R agonist UTP can induce migration of human 1321N1 cells expressing the WT P2Y2R but not a P2Y2R in which the RGD sequence was mutated to RGE (Fig. 1), a sequence change that prevents integrin interaction (1). Our results strongly suggest that interaction of the P2Y2R with αi integrins is essential for UTP-induced cell migration, and this report is the first demonstration that a GPCR can interact with an integrin to facilitate cell migration. In addition, two other lines of evidence demonstrate the involvement of αi integrins in P2Y2R-mediated cell migration. First, selective inhibition of αi integrin

FIGURE 6. P2Y2R-mediated Vav2 and Rac activation and vitronectin up-regulation require αi integrin activity. Human 1321N1 cells expressing the WT P2Y2R were incubated overnight with 10 μg/ml anti-αi or anti-αi/β1 Abs, then stimulated with 100 μM UTP for 5 min (to measure Vav2 phosphorylation [p-Vav2] and Rac GTPase activity) or 8 h (to measure vitronectin up-regulation). The data shown are representative of results from three to four experiments.

FIGURE 7. The RGD domain of the P2Y2R is required for activation of Gq but not Gαi. Membrane preparations from 1321N1 cells expressing the WT or RGE mutant P2Y2R were treated with or without 1 mM UTP and [35S]GTPγS for 15 min at 30 °C. At the termination of the assay samples were immunoprecipitated with anti-Gαq or anti-Gαi Ab, and [35S]GTPγS binding to the immunoprecipitates was quantified by liquid scintillation spectrometry. Data are the means ± S.E. of results from three experiments performed in triplicate, where the asterisk (*, p < 0.05) indicates a significant difference from UTP-stimulated control.

FIGURE 8. Role of Gq in P2Y2R-mediated cell migration, Vav2 and Rac activation, and vitronectin up-regulation. A, human 1321N1 cells expressing the WT P2Y2R were seeded into the upper chamber of Transwells and stimulated with 100 μM UTP (added to the lower chamber) in the presence or absence of 200 ng/ml PTX (added to both chambers) as indicated. Cell migration was evaluated 16 h after UTP stimulation. Data are the means ± S.E. of results from three experiments performed in triplicate, where the asterisk (*, p < 0.05) indicates a significant difference from UTP-stimulated control. B, human 1321N1 cells expressing the WT P2Y2R were pretreated with PTX for 16 h in serum-free medium then stimulated with 100 μM UTP for 5 min (to measure Vav2 and Rac activity) or 8 h (to determine vitronectin up-regulation), as indicated. The data shown are representative of results from three to four experiments. p-Vav2, phosphorylated Vav2.

FIGURE 9. Role of the P2Y2R RGD domain in UTP-induced Akt phosphorylation. Human 1321N1 cells expressing the WT or RGE mutant P2Y2R were stimulated with the indicated concentration of UTP for 10 min before analysis of Akt phosphorylation (p-Akt, A) or pretreated for 16 h with PTX before stimulation with 1 mM UTP for 10 min and analysis of Akt phosphorylation (B). Data shown are representative of results from three experiments.
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expression by antisense oligonucleotide inhibited UTP-induced cell migration of 1321N1 cells expressing the WT P2Y2R (Fig. 4B). Second, anti-αv and anti-α5β3 Abs inhibited UTP-induced migration of WT P2Y2,R-transfected cells (Figs. 5, A and B). These observations are consistent with other reports indicating that integrin activity is required for both GPCR-mediated (34, 35) and growth factor receptor-mediated cell migration (36, 37).

During cell migration integrins attach to and detach from their extracellular matrix ligands to provide the necessary traction for cell crawling (38). It also has been demonstrated that in migrating neutrophils the active form of the αβ2 integrin is concentrated at the leading edge of the cell (2, 39, 40) and that this integrin cycles between an active (i.e. extracellular matrix-bound form that is tyrosine-phosphorylated on the β3 subunit) and an inactive conformation in a calcium- and calcineurin-dependent manner (39). In the chemotaxis assays performed in the present study, the Transwell membranes were not coated with extracellular matrix proteins, and thus, we presumed that activation of the P2Y2,R induced the synthesis and secretion of extracellular matrix proteins needed to facilitate cell migration. Here, we demonstrated that UTP increased the expression of the extracellular matrix proteins vitronectin (Fig. 2A) and osteopontin (data not shown) in 1321N1 cells expressing the WT but not the RGE mutant P2Y2,R, suggesting that integrin interaction also is required for up-regulation of extracellular matrix proteins by the P2Y2,R. Furthermore, anti-vitronectin Abs inhibited UTP-induced migration of 1321N1 cells expressing the WT receptor (Fig. 2B). From these observations it seems likely that P2Y2,R-mediated cell migration is enhanced by the synthesis and secretion of extracellular matrix proteins that interact with αβ3/β3 integrins (15). Therefore, it also seems likely that the P2Y2,R and extracellular matrix proteins compete for binding to αβ3/β3 integrins, and this competition may facilitate the cyclic attachment/detachment of the cell to the extracellular matrix that is necessary for cell migration. In support of this idea we observed that 1321N1 cells expressing the WT P2Y2,R adhered to vitronectin-coated dishes at a slower rate than untransfected cells or cells expressing the RGE mutant (data not shown). Further investigation, however, is required to establish this hypothesis.

Recent advances in the understanding of the mechanism of F-actin polymerization have shown that small GTPases like Cdc42, RhoA, and Rac are major regulators of actin dynamics (23, 41) and play a critical role in cell motility. For example, activated Cdc42 stimulates the formation of actin-rich filopodia, which promotes cell migration (42). In our experiments UTP stimulated Rac phosphorylation in 1321N1 cells expressing the WT P2Y2,R but not in cells expressing the RGE mutant P2Y2,R (Fig. 3A). Dominant-negative Rac inhibited UTP-induced migration of cells expressing the WT P2Y2,R (Fig. 3C), providing direct evidence for the involvement of Rac in P2Y2,R-mediated cell migration. The small GTPases cycle between active GTP-bound and inactive GDP-bound states depending on their regulation by GEFs. One GEF for Rac is Vav2, which is activated by tyrosine kinase-dependent phosphorylation (43), and UTP has been found to cause a strong time-dependent increase in the tyrosine phosphorylation of Vav2 in human coronary artery endothelial cells (44). In the present study Vav2 phosphorylation was stimulated by UTP in 1321N1 cells expressing the WT P2Y2,R but not in cells expressing the RGE mutant P2Y2,R (Fig. 3A), suggesting that Vav2 phosphorylation, like Rac activation, was dependent on P2Y2,R interaction with αv integrins. Furthermore, DN Vav2 completely inhibited UTP-induced Rac activation (Fig. 3B), thus establishing that Vav2 plays a crucial role in P2Y2,R-mediated Rac activation and chemotaxis. The requirement for P2Y2,R and αv integrin interaction in the activation of Vav2 and Rac was demonstrated in two other ways. First, selective inhibition of αv integrin expression with αv antisense oligonucleotide inhibited the UTP-induced activation of Vav2 and Rac (Fig. 4C). Second, UTP-induced phosphorylation of Vav2 and Rac were significantly inhibited by an overnight treatment of WT P2Y2,R-transfected cells with anti-αvβ3 Ab (Fig. 6). Thus, our results strongly suggest that Vav2 and Rac activation are required for UTP-induced cell migration, responses that are dependent upon interaction between the P2Y2,R and αv integrins.

Heterotrimeric G proteins in the Gtαi family were recently reported to be responsible for GPCR-mediated cell migration by activating RacGEFs, which in turn activate Rac (8). In this study, we utilized the [35S]GTPγS binding assay to provide direct evidence that the RGD integrin binding domain in the P2Y2,R is required for this receptor to activate Gt, but not Gt (Fig. 7). We also found that the Gt, inhibitor PTX decreased UTP-induced migration of 1321N1 cells expressing the WT P2Y2,R by 70–80% (Fig. 8A) and completely inhibited P2Y2,R-mediated Vav2 and Rac activation and vitronectin up-regulation (Fig. 8B). Taken together, these results suggest that αv integrin interaction with the P2Y2,R is required to activate Gt and initiate Gt-mediated Vav2 and Rac activation and vitronectin up-regulation, leading to increased cell migration.

These results provide novel insight into the mechanism of chemotactic signaling by a GPCR and suggest that αv integrin-based complexes can be important for allowing access to specific heterotrimeric G proteins involved in cell migration. Because αv integrins have been shown to play a critical role in cell migration and angiogenesis in vascular endothelial cells (42, 45), therapeutic strategies that disrupt P2Y2,R interaction with αv integrins could be useful in the treatment of diseases involving inflammation and angiogenesis, such as atherosclerosis, diabetes, Alzheimer disease, and cancer.

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