P2Y5 is a G\(\alpha_i\), G\(\alpha_{12/13}\) G protein-coupled receptor activated by lysophosphatidic acid that reduces intestinal cell adhesion

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Lee M, Choi S, Halldén G, Yo SJ, Schichnes D, Aponte GW. P2Y5 is a G\(\alpha_i\), G\(\alpha_{12/13}\) G protein-coupled receptor activated by lysophosphatidic acid that reduces intestinal cell adhesion. Am J Physiol Gastrointest Liver Physiol 297: G641–G654, 2009. First published August 13, 2009; doi:10.1152/ajpgi.00191.2009.—P2Y5 is a G protein-coupled receptor that binds and is activated by lysophosphatidic acid (LPA). We determined that P2Y5 transcript is expressed along the intestinal mucosa and investigated the intracellular pathways induced by P2Y5 activation, which could contribute to LPA effects on intestinal cell adhesion. P2Y5 heterologously expressed in CHO and small intestinal hBRIE 380i cells was activated by LPA resulting in an increase in intracellular calcium ([Ca\(^{2+}\)]\(_i\)) when the cells concurrently expressed G\(\alpha_{12/13}\)-exons. P2Y5 activation also increased the phosphorylation of ERK1/2 that was sensitive to pertussis toxin. Together these indicate that P2Y5 activation by LPA induces an increase in [Ca\(^{2+}\)]\(_i\) and ERK1/2 phosphorylation through G\(\alpha_i\). We discovered that P2Y5 was activated by farnesyl pyrophosphate (FPP) without a detectable change in [Ca\(^{2+}\)]\(_i\). The activation of P2Y5 by LPA or FPP induced the activity of a serum response element (SRE)-linked luciferase reporter that was inhibited by the RGS domain of p115RhoGEF, C3 exotoxin, and Y-27632, suggesting the activation of SRE reporter activity is through a G\(\alpha_{12/13}\) G protein-coupled receptor activated by LPA or FPP. In addition, only LPA transactivated the epidermal growth factor receptor, leading to an induction of ERK1/2 phosphorylation. These observations correlate with our subsequent finding that P2Y5 activation by LPA, and not FPP, reduced intestinal cell adhesion. This study elucidates a mechanism whereby LPA can act as a luminal and/or serosal cue to alter mucosal integrity.

LYSOPHOSPHATIDIC ACID (LPA) is a phospholipid derivative that can act as a potent extracellular signaling molecule through the activation of G protein-coupled receptors (GPCRs). The better known LPA receptors belong to the endothelial differentiation gene (EDG) family, designated as LPA1/EDG2, LPA2/EDG4, and LPA3/EDG7 (41). Cellular processes initiated by LPA receptor activation include neurite retraction, cytoskeletal reorganization, smooth muscle cell contraction, and cell proliferation (53, 59).

The intestinal mucosal integrity essential for the functions of healthy intestine requires the coordination of renewal and migration of epithelial cells from the crypt to villus tip where anoikis [a programmed cell death caused by the loss of integrin-mediated cell adhesion (15)] occurs (62). LPA contributes to the maintenance of the intestinal epithelial integrity (60) by regulating cellular events such as epithelia restitution and inflammation responses (6, 60, 73). The intestine is a unique tissue that is exposed to both exogenous LPA from the diet (45) and endogenous LPA from the circulation and cells in the mucosa (3, 13, 42). The EDG family receptors such as LPA1 and LPA2 are expressed in the intestine; however, specific functions of non-EDG family LPA-activated GPCRs have not been elucidated.

Recently, the activation of a family of GPCRs, with sequence similarities to the purinergic receptors (P2Y family), by LPA was reported (7, 31, 33, 44, 47–49). Some of these receptors are also activated by other agonists in addition to LPA (7, 44, 48). The multiple effects of LPA on the intestine might be explained by the presence of these non-EDG family LPA responsive GPCRs, which are capable of having their activation by LPA enhanced by the presence of other ligands to this group of receptors. Of these newly identified LPA-responsive receptors, we have observed that P2Y5 mRNA appears to be at similar levels along the proximal to distal intestine. The distribution of P2Y5 transcript along the intestinal mucosa would suggest that the activation of this receptor might be important for maintaining events involved in mucosal homeostasis.

P2Y5, originally designated as 6H1, was later renamed as P2Y5 because of its similarities to nucleotide receptors (P2Y family) and its ability to bind \(^{35}\)SADPheS (67). Nevertheless, the identity of the agonist of P2Y5 remained elusive since nucleotides did not activate second messenger systems in P2Y5 transfected cells regardless of their high binding affinities (35). The prior establishment of LPA as an agonist of the P2Y5-related GPCRs [i.e., P2Y9 and GPR93 (also named GPR92)] (7, 31, 33, 47, 48)] led to the discovery that P2Y5 is activated by LPA as well (49). However, the mechanism(s) of the biological effects resulting from the activation of P2Y5 remains to be clarified. Critical to the understanding of the role(s) that P2Y5 could play in the intestine is the determination of its downstream effector molecules upon activation.

In the present study, we demonstrate P2Y5 activation by LPA and farnesyl pyrophosphate (FPP). This activation is coupled to G\(\alpha_i\) and G\(\alpha_{12/13}\), resulting in a reduced forskolin-stimulated cAMP level, an increase in the phosphorylation of ERK1/2, an induction of serum response element (SRE)-linked reporter activity, and a reduction in small intestinal hBRIE 380i cell attachment to extracellular matrix substratum. The increase in ERK1/2 phosphorylation is through a G\(\alpha_i\) pathway and mediated by MEK1/2. The activation of SRE reporter activity is through a G\(\alpha_{12/13}\) pathway and mediated by Rho kinase (ROCK), possibly through the activation of Rho family small GTPase. This study demonstrates that P2Y5 may play an important role in intestinal cell adhesion in response to mole-
Materials. 1-Oleoyl [oleoyl-9, 10-3H(N)] lysocephosphatidic acid (47.0 Ci/mmol) and [1-3H(N)]-farnesyl pyrophosphate (26.2 Ci/mmol) were obtained from PerkinElmer Life Sciences. Oleoyl LPA (18:1-LPA), oleoyl thio phosphatase, and tetradecyl phosphate were purchased from Cayman Chemical. Stearyl LPA (18:0-LPA), palmitoyl LPA (16:0-LPA), myristoyl LPA (14:0-LPA), dioleoyl phosphatidic acid (18:1-PA), and oleoyl phosphatidylcholine (18:1-PC) were purchased from Avanti Polar Lipids. Pertussis toxin (PTX), Y-27632, 1-Oleoyl [oleoyl-9, 10-3H(N)] lysophosphatidic acid, 1-Oleoyl [oleoyl-9, 10-3H(N)] phosphatidylcholine, and 1-Oleoyl [oleoyl-9, 10-3H(N)] sphingomyelin were purchased from Calbiochem. All other reagents used in this study, including meat protein hydrolysate (peptone; a mixture of enzymatically derived peptide fragments, mostly between 120 and 1,200 Da, and free amino acids) type I, were purchased from Sigma-Aldrich unless indicated differently. The LPA used in this study was dissolved in PBS (pH 7.4) with 0.1% fatty acid-free BSA (fBSA) as a carrier.

Plasmid construction. The mitochondria-targeted acetoquin (mtAEQ), Go15, GoDgS, β2-adrenergic receptor (β2AR), P2Y5, P2Y5 tagged at the COOH terminus with the enhanced green fluorescent protein (EGFP) (P2Y5-EGFP fusion), and P2Y9 expression vectors were constructed as previously described (7). All primers used in the construction of the plasmids are listed in Table 1.

Table 1. Oligonucleotide primers used for RT-PCR analyses and cDNA cloning

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>P2Y5-EGFP</td>
<td>CGGGATCCATTATAGCTGCCATGGGAGAA</td>
<td>CTTCTTGCGGGATCCATTATAGCTGCCATGGGAGAA</td>
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<tr>
<td>RGS-p115</td>
<td>CCGCACCATGGTTGATGCTGTCTGACATGGGAGAA</td>
<td>TTCTTGCGGGATCCATTATAGCTGCCATGGGAGAA</td>
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<td>RGS-GRK2</td>
<td>CTCCGCTGCTGGAGGAGATCTGAGATGCTGAGGAGAA</td>
<td>GGGATCCATTATAGCTGCCATGGGAGAA</td>
</tr>
<tr>
<td>P2Y5</td>
<td>GGGATCCATTATAGCTGCCATGGGAGAA</td>
<td>CTTCTTGCGGGATCCATTATAGCTGCCATGGGAGAA</td>
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</table>

The primer sequences are listed in 5' to 3' direction.
The hBRIE 380i cells were transfected with the P2Y5-EGFP fusion construct by electroporation (4 μg plasmid DNA/10^6 cells). After a recovery incubation in IMDM-10% BCS under normal culture conditions for 24 h, cells were trypsinized, resuspended in phenol red-free IMDM-10% BCS media, and plated on six-well slides coated with collagen type I at a density of 10^4/well for 16 h. The images of EGFP-tagged P2Y5 were acquired by using a Zeiss 510 Meta confocal microscope and a ×63 water-dipping lens. The samples were excited by using a 488-nm argon laser line. A 505- to 550-nm barrier filter was used to filter the emission light.

Measurement of intracellular cAMP. CHO cells were electroporated with the P2Y5 expression plasmid or empty vector (6 μg of DNA/10^6 cells) and then plated in 12-well plates (5 × 10^5 cells/well) in IMDM-10% BCS. After 24 h, cells were washed three times with PBS and preincubated in HBSS/0.1% fBSA for 30 min, followed by an additional 30 min incubation in the presence of 1 mM of 3-isobutyl-1-methylxanthine (IBMX). Cells were then treated with stimuli for 7 min. The treatments were terminated by placing the cells on ice and rinsing three times with ice-cold PBS. Cells were scraped on ice with 200 μl of 0.1 M HCl. The cystosolic fraction of each sample was obtained by centrifugation (10,000 g, for 10 min at 4°C), and the cAMP concentration was determined by an enzyme immunoassay method (Cayman Chemical). Under this condition, 2.5 mM forskolin increased intracellular cAMP by 7.4 ± 1.2-fold, and 100 μM isoproterenol increased intracellular cAMP by 8.8 ± 1.3-fold in β2AR transfected cells. The interassay coefficient of variance was less than 20%, and the lowest detectable level of cAMP was 3 nM.

Ligand binding assay. hBRIE 380i cells stably transfected with P2Y5 or the empty vector were laid down in T150 flasks (4 × 10^6 cells/flask). After 24 h in IMDM-10% BCS, cells were placed on ice and scraped with 5 ml of binding buffer [20 mM Tris–Cl, pH 7.5; 1 mM EDTA; and protease inhibitor cocktail (Roche)] per T150 flask. Cell lysates were incubated on ice for 20 min and then homogenized on ice for 40 strokes/sample. The crude membrane from hBRIE 380i cells was prepared by centrifugation at 16,000 g for 30 min at 4°C after homogenization. The pellets obtained from the centrifugation, which contained cell membranes, were resuspended in ice-cold binding buffer and the protein concentration was determined by the Bio-Rad protein assay. The crude cell membrane (30 μg) was incubated with various doses of [3H]18:1-LPA or 100 nM [3H]FPP in the presence of 0.1% fBSA for 30 min at 25°C with gentle shaking. The binding reaction was stopped by placing the samples on ice, followed by a 1:10 dilution with ice-cold binding buffer containing 0.1% fBSA. The membrane bound [3H]18:1-LPA was immediately vacuum collected on a GF/C glass microfiber filter disk (Whatman). The filter was then rinsed three times with ice-cold binding buffer containing 1% BSA and dried overnight at room temperature. The radioactivity of each sample was measured in a liquid scintillation counter (Beckman). Nonspecific binding was determined in the presence of 10 μM unlabeled 18:1-LPA or FPP. The value of Kd and Bmax and the minimum effective dose in each experiment. The concentrations of each inhibitor used were lower or equal to those reported by others when performing experiments under similar conditions.

Statistical analysis. Where applicable, data were expressed as means ± SD. Statistical difference between multiple groups was determined by one-way ANOVA with Duncan’s post hoc test performed with SPSS version 11. Significance was accepted at P < 0.05. Dose-response curves were generated by using the curve fitting software GraphPad Prism version 4 (GraphPad Software).
Table 2. The inhibitors used in this study and their respective targets

<table>
<thead>
<tr>
<th>Inhibitor Name</th>
<th>Chemical Inhibitors</th>
<th>Gene Inhibitors</th>
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<tbody>
<tr>
<td>Pertussis toxin</td>
<td>Gα6 family and Gαδ6γ5myr</td>
<td>Gαi1/2</td>
</tr>
<tr>
<td>PD 98059</td>
<td>ERK1/2</td>
<td></td>
</tr>
<tr>
<td>Genistein</td>
<td>Tyrosine kinase</td>
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<tr>
<td>AG 1478</td>
<td>EGFR</td>
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<tr>
<td>RGS-p115RhoGEF</td>
<td>Gα12/13 family</td>
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<td>RGS-GRK2</td>
<td>Gαi family</td>
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<td>C3-exoenzyme</td>
<td>Rho family GTPase</td>
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<td>LY294002</td>
<td>Phosphoinositide-3 kinase</td>
<td>FTI-277</td>
</tr>
<tr>
<td>Edelfosine</td>
<td>Phosphatidylinositol-specific PLC</td>
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</tr>
<tr>
<td>Go 6976</td>
<td>PKCα, PKCβ1, PKCγ, PKD1 (PKCδ)</td>
<td>Farnesyltransferase</td>
</tr>
<tr>
<td>U-73122</td>
<td>PKD2, and PKD3 (PKCv)</td>
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</tr>
<tr>
<td>FTI-277</td>
<td>Farnesyltransferase</td>
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The inhibitors were used as described in MATERIALS AND METHODS.

RESULTS

P2Y5 transcript is expressed along the proximal-to-distal intestinal mucosa. The mRNA expression of P2Y5 in various rat tissues was quantified by semiquantitative RT-PCR, and the abundance of the P2Y5 transcripts was similar in all tested tissues (i.e., brain, heart, lung, kidney, pancreas, liver, stomach, small and large intestine) (data not shown). In the intestine, P2Y5 mRNA is expressed in mcosa from the duodenum to the ileum at similar levels (Fig. 1A), with a twofold higher expression in the villus tip than in the crypt region (Fig. 1B). This is consistent with the higher P2Y5 expression in differentiated hBRIE 380i cells (Fig. 1C). Both LPA and FPP are found in the circulation (41, 55), and LPA is also found in food (45). The villus tip expression of P2Y5 provides a convenient location for the receptor to have access to agonists that are present in both the apical and basolateral plasma membranes. Although the mRNA levels of GPCRs can represent the protein levels in intestinal epithelial cells (e.g., the sphingosine-1-phosphate receptor’s mRNA levels reflect the protein levels in T cell (65)), the possibility that P2Y5 mRNA levels might not reflect the protein levels in the intestine remains to be determined.

The hBRIE 380i cells express EGFP-tagged P2Y5 on the plasma membrane. LPA could exert its effects both intra- and extracellularly (39). The cellular localization of the LPA responsive receptors would help in determining the initial site of regulation of the signaling pathways responsive to LPA. Therefore, the localization of heterologously overexpressed P2Y5 tagged with EGFP at its COOH terminus was determined in hBRIE 380i cells. Fluorescence confocal microscopy revealed expression of the receptor on the surface plasma membrane (Fig. 2A). These data suggest that the observed P2Y5 activation in our subsequent studies occurred at the cell surface.

Activation of P2Y5 by LPA in CHO and hBRIE 380i cells leads to Gαi-mediated signaling pathways. It has been observed that LPA mediated the induction of CRE reporter activity in P2Y5-overexpressing CHO cells (49). This induction occurred in a dose-responsive manner that correlated with increased LPA binding in P2Y5-overexpressing HEK cells. It was, therefore, proposed that P2Y5 was an LPA-responsive GPCR (49). Since CRE reporter activity is induced by more than one family of Gα (40), the Gα protein coupling preferences of P2Y5 remained unclear. All known LPA responsive GPCRs can couple with multiple families of Gα (41); therefore, the Gα coupling preferences of P2Y5 in response to LPA were first examined by using an aequorin-based calcium assay to characterize the downstream signal cascades that are activated by P2Y5. Although both the 1-acetyl and 2-acetyl LPA isomers exist in the circulation in vivo (51, 63), 2-acetyl LPA is not stable under physiological conditions (51). Therefore, we used the 1-acetyl isomer in these studies.

We found that the [Ca2+]i, in P2Y5-overexpressing CHO cells was not different from the empty vector transfected CHO cells upon stimulation by oleoyl LPA (18:1-LPA), which is similar to what was reported by others (47), suggesting that P2Y5 is not a Gαi-coupled GPCR. The activation of Gαi and Gαq-coupled GPCRs are not usually associated with detectable changes in [Ca2+]i. By cotransfecting a promiscuous Gαi, in this case Gα15 or Gαδ6γ5myr (30), the activation of Gαi or
Gαi-coupled GPCRs, respectively, can be quantified in a calcium assay, thus allowing changes in [Ca²⁺]i to be used as an indicator for the activation of Gαs-, Gαi-, and Gαq-coupled GPCRs. In the presence of Gα6qi5myr, a dose-dependent rise in [Ca²⁺]i in response to 18:1-LPA (EC₅₀ = 195.3 nM) was higher in P2Y5-overexpressing CHO cells than in the cells that were transfected with the vector and Gα6qi5myr (Fig. 2B). The enhanced induction of [Ca²⁺]i in response to LPA in P2Y5 and Gα6qi5myr transfected cells was abolished in cells that were pretreated with 80 ng/ml PTX (a specific inhibitor of Gαi family and Gα6qi5myr) (data not shown). This confirmed that the cotransfected Gα6qi5myr was the mediator for enhancing the induction of [Ca²⁺]i. Cotransfecting Gα15 only enhanced the [Ca²⁺]i increase in P2Y5 transfected cells at micromolar levels of 18:1-LPA (Fig. 2B). Experiments repeated in hBRIE 380i cells also demonstrated that Gα6qi5myr

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Fig. 2. Lysophosphatidic acid (LPA)-activated P2Y5 changes intracellular Ca²⁺ and cAMP levels. A: the localization of P2Y5 tagged with EGFP on the cell surface plasmalemma was visualized by laser scanning confocal microscopy. The scale bar represents 10 μm. B: Chinese hamster ovary (CHO) cells were transfected with the mitochondria-targeted aequorin (mtAEQ) plus P2Y5 or P2Y9 cDNA and the empty vector (●), Gα6qi5myr (□), or Gα15 (△). After a 20-h recovery, cells were loaded with coelenterazine-H for 2 h during a gentle rolling. The intracellular Ca²⁺ concentration ([Ca²⁺]i) in response to various doses of LPA, as indicated in the figure, was assayed as described in MATERIALS AND METHODS. Integrated RLU represents the integrated fractional relative light units (RLU) value for a 30-s period after the introduction of LPA. Data points are means ± SD (n = 4–8). C: hBRIE 380i cells were transfected with mtAEQ, Gα6qi5myr, and either P2Y5 (■) or the empty vector (○). The changes in [Ca²⁺]i were assayed as described in B. Data points are means ± SD (n = 4–8). D: CHO cells transfected with mtAEQ, P2Y5, and Gα6qi5myr cDNA were loaded with coelenterazine-H. [Ca²⁺]i was assayed with various doses of 18:1-LPA (■), 18:1-PA (●), 18:0-LPA (▲), 16:0-LPA (▼), and 14:0-LPA (○). Normalized integrated RLU represents the integrated RLU value obtained from P2Y5 transfected cells subtracted from that of empty vector-transfected cells. Data points are means ± SD (n = 3). E: CHO cells were transfected with P2Y5 cDNA or the empty vector. After a 48-h recovery, cells were treated with 2.5 μM of forskolin/1 mM IBMX in the presence or absence of 1 μM LPA for 7 min. Intracellular cAMP level was measured by enzyme immunoassay as described in MATERIALS AND METHODS. Percent cAMP is the ratio of cAMP concentration in LPA- and forskolin-treated cells to that in forskolin-treated cells, which is arbitrarily set to 100. Bars are means ± SD (n = 3). *P < 0.05 vs. corresponding color in bar 1.
Table 3. Phospholipids’ and other related compounds’ potencies are determined by monitoring [Ca^{2+}]i induction in CHO cells

<table>
<thead>
<tr>
<th>Name</th>
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<th>E_{max}</th>
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<tr>
<td>18:1 LPA</td>
<td>0.16</td>
<td>0.45</td>
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<tr>
<td>18:0 LPA</td>
<td>4.56</td>
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</tr>
<tr>
<td>16:0 LPA</td>
<td>0.23</td>
<td>0.34</td>
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<tr>
<td>14:0 LPA</td>
<td>61.46</td>
<td>0.42</td>
</tr>
<tr>
<td>18:1 PA</td>
<td>9.62</td>
<td>0.45</td>
</tr>
<tr>
<td>Oleoyl thiophosphate</td>
<td>9.32</td>
<td>0.50</td>
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</table>

Chinese hamster ovary (CHO) cells were transfected with mAEQ. P2Y5 cDNA (or the empty vector) and G_{α_{q5myr}}. P2Y5-specific intracellular Ca^{2+} concentration ([Ca^{2+}]i) induction was determined by subtracting the change in the [Ca^{2+}]i level in vector-transfected cells from that in P2Y5-transfected cells.

was necessary for the detection of the P2Y5-specific [Ca^{2+}]i response (EC_{50} = 1.59 μM; Fig. 2C), suggesting that P2Y5 might be an LPA-responsive G_{α_{q}} (and not G_{α_{s}})-coupled GPCR.

To investigate whether the activation of P2Y5 was only specific to the 18:1-LPA, the potencies of other related phospholipids on P2Y5 activation were tested in CHO cells cotransfected with G_{α_{q5myr}} (Fig. 2D). Listed in Table 3 are the EC_{50} values of the tested phospholipids. The rank of potency was 18:1-LPA > 16:0-LPA > 18:0-LPA > 18:1-PA > 14:0-LPA. In all subsequent experiments, unless indicated otherwise, the 18:1-LPA was used as the agonist for P2Y5. Some phospholipid responsive receptors are also activated by changes in proton concentration ([H+]i), such as OGR1, GPR4, G2A, and TDAG8 (64). Therefore, the effects of varying [H+]i on the activation of P2Y5 in CHO cells cotransfected with G_{α_{q5myr}} were assayed by the calcium mobilization assay. Different [H+]i (pH 6.8, pH 7.2, pH 7.8, or pH 8.2) had no effect on the [Ca^{2+}]i in the presence and absence of 10 μM LPA. Listed in Table 4 are the tested compounds that did not activate P2Y5 in CHO cells cotransfected with G_{α_{q5myr}}.

For comparison, we also explored the effects of 18:1-LPA on the activation of P2Y9, an LPA-responsive “P2Y5-like” GPCR that couples to both G_{α_{s}} and G_{α_{q}} families (25, 32), using the aequorin-based calcium assay in the presence or absence of the promiscuous G_{α_{s}} proteins. Changes in [Ca^{2+}]i in response to 18:1-LPA in P2Y9-overexpressing CHO cells were enhanced with cotransfection of either G_{αs} (EC_{50} = 153.3 nM) or G_{α_{q5myr}} (EC_{50} = 166 nM) (Fig. 2B). The EC_{50} values of the [Ca^{2+}]i induction of P2Y5 and P2Y9 cells were comparable, verifying the LPA responsiveness of P2Y5. In addition, the EC_{50} value of P2Y5 was similar to the LPA receptors of the EDG family, such as LPA3 (EC_{50} = 214 nM) (11). Because GPR93 is also activated by protein hydrolysate, we tested for a similar activation of P2Y5. As also observed for P2Y9 (9), protein hydrolysate (up to 50 mg/ml) did not enhance [Ca^{2+}]i in CHO cells transfected with P2Y5 and G_{α_{q5myr}}.

LPA-activated P2Y5 reduces forskolin-stimulated [cAMP]i in CHO cells. The preference of P2Y5 to couple to G_{α_{q5myr}} over G_{αs} suggests that P2Y5 prefers coupling with the G_{αq} over the G_{αs} family. In CHO cells, LPA (up to 10 μM) did not significantly increase intracellular cAMP level ([cAMP]i) in either vector or P2Y5 transfected CHO cells (data not shown), suggesting that P2Y5 activation was not coupled to G_{αs}. A 7-min treatment of LPA (1 μM) significantly reduced the forskolin (2.5 μM)-elevated [cAMP]i in P2Y5-transfected cells by 44% (Fig. 2E). These results indicated that P2Y5 activation was coupled to the G_{αq}, not to the G_{αs} family, which confirmed the results of the calcium mobilization assay. Consistent with this finding, LPA (up to 10 μM) did not induce CRE-linked luciferase reporter activity in hBRIE 380i cells (data not shown).

P2Y5 activation by LPA, in hBRIE 380i cells, induces PTX-sensitive ERK1/2 phosphorylation. LPA treatment of cell lines, such as hBRIE 380i, can induce an increase in ERK1/2 phosphorylation through G_{αq}-mediated signal cascades (7, 10, 41). Therefore, we explored the effects of P2Y5 activation on ERK1/2 phosphorylation in hBRIE 380i cells. LPA at 10 μM induced a rapid phosphorylation of ERK1/2 that reached a maximum at 4 min followed by a gradual decrease (Fig. 3A). P2Y5 overexpression in hBRIE 380i cells enhanced the phosphorylation of ERK1/2 in response to LPA, the effects of various LPA concentrations on ERK1/2 phosphorylation were examined at 4 min. P2Y5 specific enhancement of ERK1/2 phosphorylation was dose dependent and peaked at 5 μM (Fig. 3B), which was in a range similar to the EC_{50} value obtained by the aequorin-based calcium assay in hBRIE 380i cells. The phosphorylation of ERK1/2 in response to LPA activated P2Y5 was blocked by 50 μM PD98059, an inhibitor of MEK1/2 (Fig. 3C). PTX at 80 ng/ml almost eliminated the P2Y5 mediated increase in ERK1/2 phosphorylation, suggesting that the activation of the G_{αq} family was required for the P2Y5 induction of ERK1/2 phosphorylation (Fig. 3C).

EGFR transactivation is involved in the P2Y5-mediated ERK1/2 phosphorylation. LPA can also increase ERK1/2 phosphorylation through the transactivation of receptor tyrosine kinase (RTK) by LPA-responsive GPCRs (69); therefore, we examined the potential involvement of tyrosine kinase in the phosphorylation of ERK1/2. Genistein, a tyrosine kinase inhibitor (1), at 10 μM significantly decreased P2Y5-induced ERK1/2 phosphorylation (Fig. 3D), suggesting the involvement of tyrosine kinase. One member of the RTK family that is transactivated by LPA receptors and expressed endogenously in hBRIE 380i cells (Fig. 3D, inset) is EGFR. A

Table 4. Tested compounds that do not elevate [Ca^{2+}]i in P2Y5- and G_{α_{q5myr}}-cotransfected CHO cells

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>18:1-PC</td>
<td>NE ≤ 100 μM</td>
</tr>
<tr>
<td>Tetradecyl phosphate*</td>
<td>NE ≤ 10 μM</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>NE ≤ 20 μM</td>
</tr>
<tr>
<td>Platelet activating factor*</td>
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<td>Geranyl diphosphates</td>
<td>NE ≤ 10 μM</td>
</tr>
<tr>
<td>Farnesol</td>
<td>NE ≤ 10 μM</td>
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CHO cells were transfected with mAEQ, P2Y5 cDNA (or the empty vector), and G_{α_{q5myr}}. P2Y5-specific [Ca^{2+}]i induction was determined as described in Table 3. PC, phosphatidylcholine. NE, not effective. *Tested for antagonism against lysophosphatidic acid (LPA).
specific EGFR inhibitor (AG 1478) at doses from 5 to 50 nM significantly inhibited the P2Y5-induced ERK1/2 phosphorylation in response to LPA (Fig. 3D), suggesting that LPA-activated P2Y5 could transactivate EGFR through the Goq family, resulting in an increase of ERK1/2 phosphorylation in hBRIE 380i cells.

**LPA induces SRE-linked luciferase reporter activity in P2Y5-overexpressing cells.** Most LPA-responsive GPCRs can couple to Go12/13 (33, 41, 71). The aequorin-based calcium assay of cells cotransfected with promiscuous Go proteins cannot be used to determine the coupling of the Go12/13 family by activated GPCRs because a construct of Goq that has been shown to couple to GPCRs that only associate with Go12/13 family has yet to be developed. Therefore, changes of SRE reporter activity [a commonly used indicator for Goq12/13 family activation (52)] in response to LPA was determined in P2Y5-overexpressing cells. SRE reporter activity can also be induced by the activation of Goq12/13-coupled GPCRs. Since P2Y5 does not couple with Goq12/13, the induction of SRE reporter activity by P2Y5 activation would indicate the activation of a Goq12/13-mediated signal pathway.

Treatment of P2Y5-transfected hBRIE 380i cells with LPA for 6 h increased SRE-linked reporter activity in a dose-dependent manner. At a 10 μM concentration, LPA increased the SRE reporter activity by 18.2-fold (compared with a 4.4-fold increase in the empty vector transfected cells) (Fig. 4A). The EC50 value was 3.4 μM for 18:1-LPA-induced SRE reporter activity that was similar to the EC50 value determined by the aequorin-based calcium assay in hBRIE 380i cells. A dose lower than the EC50 value of 18:1-LPA, 1 μM, was used to compare the potencies of different LPAs on the SRE reporter activation. The 18:1-LPA was still the most effective agonist for the SRE reporter induction compared with the other LPAs tested (Fig. 4B), and the rank of potencies among different LPAs for the SRE reporter induction was similar to the rank of potencies determined by the aequorin-based calcium assay. These studies indicate that P2Y5 is coupled to the Goq12/13 family proteins.

**FPP increases SRE reporter activity through P2Y5, in hBRIE 380i cells, without activating Goq-mediated signal cascades.** FPP is an endogenous ligand that acts as an antagonist to the activation of EDG family receptors by LPA (36) and an agonist to GPR93 activation by LPA (48). FPP did not act as an antagonist to LPA on the induction of [Ca2+]i in P2Y5 and Goq12q5myr-cotransfected CHO cells or to LPA-induced SRE reporter activity in P2Y5-transfected hBRIE 380i cells (data not shown). FPP alone (up to 10 μM) did not induce [Ca2+]i flux in CHO or hBRIE 380i cells overexpressing P2Y5 with (or without) Goq12/13 or Goq12q5myr and ERK1/2 phosphorylation and the CRE reporter activity in both vector- and P2Y5-transfected

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**Fig. 3.** LPA induces a pertussis toxin (PTX)- and AG 1478-sensitive increase in ERK1/2 phosphorylation in P2Y5-overexpressing hBRIE 380i cells. **A:** hBRIE 380i cells stably transfected with either P2Y5 cDNA (hBRIE 380i-P2Y5) or empty vector (hBRIE 380i-vector) were treated with 10 μM LPA over various time periods as indicated in the figure. Time-dependent change in ERK1/2 phosphorylation is depicted, and the levels of ERK1/2 phosphorylation were determined as described in MATERIALS AND METHODS. **B:** hBRIE 380i-P2Y5 or hBRIE 380i-vector cells were treated with various doses of LPA as indicated in the figure for 4 min. Depicted is a representative image. **C:** effects of pertussis toxin (80 ng/ml) and PD98059 (50 μM) on the ERK1/2 phosphorylation induced by 5 μM LPA for 4 min in hBRIE 380i-P2Y5 or hBRIE 380i-vector cells were determined by Western blot and densitometry analysis as described in MATERIALS AND METHODS. The relative quantitative values are shown in the histogram. Fold induction represents the ratio of phospho-ERK1/2 band intensity to the band intensity of total ERK1 from each treatment normalized to that of the control (bar 1; 5 μM LPA alone without inhibitors), which is arbitrarily set to 100. Bars are means ± SD (n = 3). *P < 0.05 vs. corresponding color in bar 1. **D:** effects of genistein (GE; 1 or 10 μM) and AG 1478 (AG; 1, 5, or 50 nM) on 5 μM LPA-induced ERK1/2 phosphorylation were determined in hBRIE 380i-P2Y5 and hBRIE 380i-vector cells by Western blot and densitometry analysis as described in C. Percent inhibition is the ratio of the fold induction from P2Y5 transfected cells to empty vector transfected cells normalized to that of the control (bar 1; 5 μM LPA alone without inhibitors), which is arbitrarily set to 100. Bars are means ± SD (n = 3). *P < 0.05, vs. bar 1. Inset: the endogenous expression of EGFR in hBRIE 380i cells was determined by Western blot as described in MATERIALS AND METHODS. Depicted is a representative image, and the arrow indicates the molecular mass of 170 kDa.
hBRIE 380i cells (data not shown). However, FPP and geranylgeranyl diphosphate (GGPP) at 5 μM significantly induced SRE reporter activity in P2Y5-transfected hBRIE 380i cells by 5.2- and 2.9-fold, respectively (Fig. 4C), suggesting that FPP and GGPP could activate the G12/13 family through P2Y5. A dose-response curve was determined for FPP on the induction of SRE reporter activity by using vector- and P2Y5-transfected hBRIE 380i cells (Fig. 4D). The EC50 value for the induction of SRE reporter activity in response to FPP in cells overexpressing P2Y5 was 2.9 μM. These results suggest that P2Y5 expressed in hBRIE 380i cells was able to activate only the G12/13 family in response to FPP.

The induction of SRE reporter activity in P2Y5-overexpressing cells is mediated through G12/13 family, Rho family GTPase, and Rho kinase. The regulator of G protein signaling (RGS) domain of p115RhoGEF binds and enhances the intrinsic GTPase activity of the G12/13 family, leading to the deactivation of the G12/13 family (16). Because RGS inhibits the G12/13-mediated SRE reporter transactivation in HeLa cells (58), we investigated the effects of the RGS domain of p115RhoGEF on the induction of SRE reporter activity by LPA and FPP in HeLa cells overexpressing P2Y5 to determine the contribution of G12/13 family in P2Y5 signal transduction. Cotransfection of the RGS domain of p115RhoGEF with P2Y5 in HeLa cells significantly reduced 65% of the LPA induced SRE reporter activity (Fig. 5A), since the RGS domain of GRK2 targets the Gαq but not the G12/13 family (16). These data verified that the induction of SRE reporter activity in response to LPA-activated and FPP-activated P2Y5 was mediated by the G12/13 family.

Activation of the G12/13 family can induce the expression of SRE regulated genes through the activation of Rho and ROCK (9, 52). Overexpression of C3 exoenzyme [a toxin that inactivates Rho A, B, and C small GTPases (68)] significantly reduced the effects of 10 μM LPA and 5 μM FPP on the SRE reporter activity in P2Y5-transfected hBRIE 380i cells by 50 and 57%, respectively (Fig. 5B, bar 1 vs. bar 2). A specific inhibitor of ROCK, Y-27632, at doses from 1 to 10 μM, significantly reduced the induction of SRE reporter activity in response to LPA in the P2Y5-transfected hBRIE 380i cells (Fig. 5C). Y-27632 at 1 μM (the lowest dose producing significant inhibition of the LPA-induced SRE reporter activity) also significantly reduced the FPP-induced SRE reporter activity in the P2Y5-transfected hBRIE 380i cells (Fig. 5C). This data suggests that the Rho-ROCK pathway was involved in the transactivation of SRE reporter activity in response to LPA- and FPP-activated P2Y5 in hBRIE 380i cells. During the preparation of this manuscript, Yanagida et al. (72) reported that LPA activates the G12/13 Rho signaling pathways in P2Y5-overexpressing B103 rat neuroblastoma cells.

Phosphoinositide-3 kinase (PI3K) and p38 MAP kinase (MAPK) are downstream effectors of the G12/13 family that can mediate the induction of the SRE reporter activity (46, 66). Treatments with LY294002 (a PI3K inhibitor) and SB202190
induction of the SRE reporter activity might be mediated in a dose-dependent manner. This suggests the involvement of [Ca\(^{2+}\)]-related signal pathways (Fig. 5C). However, treatments of 2 \(\mu\)M Go\(_6976\) and 20 \(\mu\)M edelfosine did not affect the 5 \(\mu\)M FPP-induced SRE reporter activity in P2Y5-overexpressing hBRIE 380i cells, suggesting that only LPA-activated P2Y5 could initiate [Ca\(^{2+}\)]-related signal pathways that lead to the induction of SRE reporter activity.

ERK1/2 is a downstream signal molecule that can mediate the induction of SRE reporter activity and can be activated by LPA. We observed that P2Y5 activation by LPA increased ERK1/2 phosphorylation through a PTX-sensitive pathway (Fig. 3). PD98059 at 50 \(\mu\)M did not inhibit the P2Y5-mediated SRE transactivation (in response to LPA and FPP) in hBRIE 380i cells (data not shown). This result was consistent with data demonstrating that PTX treatment did not block LPA- and FPP-induced SRE reporter activity in P2Y5-overexpressed hBRIE 380i cells (data not shown).

FPP can be used metabolically as a source for 15 carbon isoprenyl lipid during the protein farnesylation process catalyzed by farnesyl transferase (FTase) (5). Some members of the Rho GTPase family are farnesylated, which affects the function of small GTPases such as RhB (5). We tested whether the farnesylation of small GTPases could account for increased activity of FPP activated P2Y5. Treatment with an FTase inhibitor, FTI-277 (10 \(\mu\)M), did not alter the effects of FPP and LPA on the transactivation of SRE reporter in hBRIE 380i cell overexpressing P2Y5 (data not shown), suggesting that protein farnesylation was not likely to be involved in P2Y5-mediated SRE transactivation. A summary of our results for LPA- and FPP-induced signaling pathways by P2Y5 activation is shown in Fig. 6.

LPA reduces hBRIE 380i cell adhesion through activation of P2Y5. LPA can modulate intestinal epithelial cell adhesion and deadhesion (or motility) processes through Go12/13-coupled GPCRs (22, 26, 60, 70). Therefore, we examined the effects of LPA activated P2Y5 on the hBRIE 380i cell adhesion. A treatment with LPA (at 1 and 10 \(\mu\)M) significantly reduced the adhesiveness of P2Y5-transfected hBRIE 380i cells compared with vector-transfected cells (Fig. 7A), indicating that LPA-activated P2Y5 could reduce hBRIE 380i cell adhesion. The extent of the decrease in adhesion was similar to what was observed with hBRIE 380i cells exposed to NPY and PYY (34).

The activation of EGFR can result in the modulation of cell adhesion, migration through the activation of p38 MAPK, and alteration of the interaction between E-cadherin and the actin cytoskeleton (12, 20, 38). Since LPA effects can be mediated by EGFR activation (21), we tested whether LPA-activated P2Y5 might also reduce cell adhesion via transactivation of EGFR. AG 1478 at 50 and 250 nM displayed a significant and dose-dependent inhibition of the reduced adhesion observed in P2Y5-overexpressing cells treated with LPA (Fig. 7B), suggesting the involvement of EGFR.

The [Ca\(^{2+}\)]-related signal pathways (e.g., the activation of PKC and PLC) are often involved in the regulation of cell adhesion and migration (29, 50). We therefore tested whether changes in [Ca\(^{2+}\)] were involved in reduction of intestinal cell adhesion resulting from LPA activation of P2Y5. The presence of 1 \(\mu\)M Go\(_6976\) or 10 \(\mu\)M edelfosine significantly inhibited the LPA-mediated reduction of adhesion in P2Y5-overexpressing (a p38 MAPK inhibitor) significantly reduced LPA-mediated induction of SRE reporter activity in P2Y5-transfected hBRIE 380i cells, in a dose-dependent manner, up to 75 and 90%, respectively (Fig. 5C) and suggested the involvement of P13K and p38 MAPK in P2Y5 signaling. However, the 5 \(\mu\)M FPP induction of SRE reporter activity observed in P2Y5-overexpressing hBRIE 380i cells was insensitive to both LY294002 (5 \(\mu\)M) and SB202190 (20 \(\mu\)M), which was arbitrarily designated as 1. Bars are means ± SD (n = 3). *P < 0.05 vs. corresponding color in bar 1. \(\bullet\) 0.05 vs. corresponding bar 2. *P < 0.05 vs. corresponding color in bar 3. A: HeLa cells were transfected with SRE-linked luciferase reporter plasmid plus vector or P2Y5 cDNA, with or without the RGS domains from p15RhGEF or GRK2, and treated with 10 \(\mu\)M LPA or 5 \(\mu\)M FPP for 6 h. Fold induction is the ratio of the value from each sample to the value from the control (open bar 1), which was arbitrarily designated as 1. Bars are means ± SD (n = 3). *P < 0.05 vs. corresponding color in bar 1. B: hBRIE 380i cells were transfected with SRE-linked luciferase reporter plasmid plus vector or P2Y5 cDNA, with or without the C3 exotoxin cDNA, and treated with 10 \(\mu\)M LPA or 5 \(\mu\)M FPP for 6 h. Percent inhibition is the fold induction of P2Y5 transfected cells to empty vector transfected cells normalized to that of the control (bar 1 of the corresponding color; 10 \(\mu\)M LPA or 5 \(\mu\)M FPP alone), which is arbitrarily set to 100. Bars are means ± SD (n = 3). *P < 0.05, relative to corresponding color in bar 1. C: effects of Y-27632 (Y27; 0.1, 1, or 10 \(\mu\)M), LY 294002 (LY; 5, 10, or 50 \(\mu\)M), SB 202190 (SB; 2, 10 or 20 \(\mu\)M), Go 6976 (Go; 0.2, 1 or 2 \(\mu\)M), or edelfosine (ED; 5, 10, or 20 \(\mu\)M) on the induction of SRE reporter in P2Y5-overexpressing hBRIE 380i cells in response to 10 \(\mu\)M LPA or 5 \(\mu\)M FPP for 6 h was determined as described in MATERIALS AND METHODS. Percent inhibition is as described in B. Bars are means ± SD (n = 3). *P < 0.05, relative to bar 1.
P2Y5 is activated by LPA and reduces cell adhesion

Fig. 6. Potential LPA- and FPP-initiated intracellular signaling pathways in hBRIE 380i cells overexpressing P2Y5. Potential LPA (left)- and FPP (right)-initiated intracellular signaling cascades through P2Y5 are illustrated in this diagram. LPA-activated P2Y5 can initiate the activation of Goi and Goi1213 families, leading to increased phosphorylation of ERK1/2 (through MEK) that is sensitive to EGFR inhibitor and SRE reporter activity that is sensitive to inhibitors targeting Rho small GTPase, ROCK, P38 MAPK, PI3K, PKC, and PLC. FPP can also activate P2Y5 and activate Goi1213 but not Goi family-mediated signal pathways. Furthermore, unlike LPA, the FPP-activated P2Y5-induced SRE reporter activity is sensitive to inhibitors targeting Rho small GTPase and ROCK but not P38 MAPK, PI3K, PKC, and PLC.

Fig. 7. LPA- and FPP-activated P2Y5 reduces hBRIE 380i cell adhesion. A: adhesiveness of hBRIE 380i-P2Y5 and hBRIE 380i-vector cells to collagen I-coated wells in the presence or absence of LPA was determined as described in MATERIALS AND METHODS. Percent adhesion is the ratio of the value from each sample to the value from the control (no LPA treatment), which was arbitrarily designated as 100. Data points are means ± SD (n = 3). %P < 0.05, relative to value of hBRIE 380i-vector cells of the same treatment. B: effect of AG1478 (5, 50, or 250 nM), Goi6976 (Go; 0.2, 1 or 2 μM), or edelfosine (ED; 5, 10, or 20 μM) was determined by pretreating cells with each inhibitor for 2 h. Ten μM LPA was used as a stimulus. Bars are means ± SD (n = 3). Percent adhesion is as described in A. %P < 0.05, relative to bar 1 (10 μM LPA treatment without inhibitors). C: adhesiveness of hBRIE 380i-P2Y5 and hBRIE 380i-vector cells to collagen I-coated wells in the presence or absence of FPP was determined as described in MATERIALS AND METHODS. Bars are means ± SD (n = 3). %P < 0.05, relative to the value from hBRIE 380i-vector cells for the same treatment.
FPP did not function as an antagonist of LPA in the calcium mobilization assay (Table 4), but FPP could activate SRE reporter activity, suggesting that FPP and LPA might interact with different structural domains of P2Y5. Therefore, the competition binding experiments with FPP against \(^{3}H\)18:1-LPA might not have revealed whether FPP could directly interact with P2Y5; hence, the binding of \(^{3}H\)FPP to hBRIE 380i cells overexpressing P2Y5 was examined. At 100 nM FPP, membrane preparations from hBRIE 380i cells transfected with P2Y5 retained a small but significantly higher (87.6%) amount of \(^{3}H\)FPP compared with the empty vector and 30 \(\mu\)g of the membrane fraction was incubated with various doses of \(^{3}H\)18:1-LPA for 30 min at 25°C with gentle shaking. Nonspecific binding was determined in the presence of 10 \(\mu\)M nonradiolabeled 18:1-LPA. The specific binding was calculated by subtracting the nonspecific binding from the total binding. Data points are means \(\pm\) SD (n = 3). The value of apparent \(K_d\) and \(B_{max}\) were calculated as described in MATERIALS AND METHODS. B: competition between 10 nM \(^{3}H\)18:1-LPA and various doses of unlabeled ATP binding to P2Y5-overexpressed hBRIE 380i membrane was determined as described in MATERIALS AND METHODS. Nonspecific binding was determined as described in A. Percent binding is the ratio of 10 nM \(^{3}H\)18:1-LPA specific binding to plasma membrane in the presence of the competitors to that without the competitors, which is arbitrarily set to 100. Bars are means \(\pm\) SD (n = 3). C: plasma membrane was isolated from hBRIE 380i cells stably transfected with P2Y5 or the empty vector, and 30 \(\mu\)g of the membrane fraction was incubated with 100 nM of \(^{3}H\)FPP. The experiment proceeded as described in A. Bars are the means of counts per minute (cpm) \(\pm\) SD (n = 3). *P < 0.05 vs. the open bar.

DISCUSSION

The epithelial cells lining the intestinal lumen are exposed to molecules from the external environment through their apical surface, as well as molecules from peripheral tissues through their basolateral surface. These molecules can modulate events that are essential for the maintenance of mucosal functional and structural integrity such as cell migration along the crypt-to-villus axis, axial and spatial differentiation, and anoikis. P2Y5 could act as a receptor that helps coordinate environmental and systemic cues on intestinal cell activities because its ligand, LPA, can be present in the intestinal lumen and also released from peripheral sources. Although P2Y5 transcript was observed to be distributed throughout the intestinal mucosa, the expressed receptor could exert a specific regional effect along the proximal to distal intestine, or along the crypt-to-villus axis, by differentially responding to more than one agonist at a given time.

We determined that FPP is also an agonist of P2Y5, thus making this receptor the second known cell surface receptor activated by FPP after GPR93 (48). FPP is an intermediate in the isoprenoid biosynthetic pathway (23), also present in the presence of LPA and FPP in vivo might inhibit the activity of LPA2 and LPA3 (36). Unlike with LPA, the treatment of P2Y5-overexpressing cells with FPP did not result in an induction of [Ca\(^{2+}\)] mobilization and ERK1/2 phosphorylation.

Both LPA and FPP activation of P2Y5 induced the Rho GTPase, ROCK pathway, and SRE reporter activity through the coupling of \(\alpha_{12/13}\); however, LPA and FPP might utilize different downstream effectors of SRE reporter activity. FPP-induced SRE reporter activity was not affected by inhibitors targeting p38 MAPK, PI3K, PLC, or PKC. Therefore, despite being P2Y5 agonists, LPA and FPP might be able to elicit distinct effector responses. Furthermore, the simultaneous presence of LPA and FPP in vivo might inhibit the activity of LPA2 and LPA3, but not that of P2Y5 and GPR93. Our finding presents an additional point of regulation for the specific effects of P2Y5 activation may not only be dependent on the expression pattern of the receptor but also on the combination of agonists at a given time.

Recently, Hypotrichosis simplex (a group of hereditary isolated alopecia) was correlated to a truncated mutation of P2Y5 by genomewide linkage analysis (49). LPA treatments of

Table 5. Competition between an LPA analog and \(^{3}H\)18:1-LPA determined by binding experiments using P2Y5 overexpressing hBRIE 380i cell membrane

<table>
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<th>Name</th>
<th>(K_i) (nM)</th>
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<tr>
<td>18:1 LPA</td>
<td>25</td>
</tr>
<tr>
<td>18:0 LPA</td>
<td>43</td>
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<tr>
<td>16:0 LPA</td>
<td>66</td>
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The specific binding of 10 nM \(^{3}H\)18:1-LPA in the presence or absence of each of the competitors and the \(K_i\) values were determined as described in the MATERIALS AND METHODS.
human P2Y5-overexpressing CHO cells led to a PTX-insensitive induction of CRE reporter activity and did not reduce forskolin-elevated CRE reporter activity. It was therefore concluded that P2Y5 activation by LPA was coupled to Goα, and not to Goi (49). However, CRE can be activated by both [Ca\textsuperscript{2+}], and cAMP-mediated pathways (2). Following LPA activation of P2Y5 in CHO cells, we detected neither changes in [Ca\textsuperscript{2+}], even with coexpression of Go\textsubscript{i15} (a G protein that can enhance [Ca\textsuperscript{2+}], mobilization by activated GPCRs coupled to Go\textsubscript{i}), nor increases in intracellular cAMP levels, which indicates that P2Y5 is likely not coupled to Go\textsubscript{i}. However, a possibility remains that P2Y5 activation might result in a latent increase in [Ca\textsuperscript{2+}], that could modulate CRE reporter activity. We observed that P2Y5-induced SRE reporter activity was sensitive to both PI-PLC and PKC inhibitors, suggesting the involvement of a Go\textsubscript{i2/13}-linked PLC isoform, such as PLC\textgamma (52), in a delayed elevation of [Ca\textsuperscript{2+}], following LPA stimulation.

The median effective concentration (i.e., EC\textsubscript{50} values) of P2Y5 agonists is not strictly correlated to the strength of ligand binding (i.e., K\textsubscript{d} values) to the receptor. P2Y5 exhibited a higher affinity to 18:0-LPA than to 16:0-LPA, but 16:0-LPA was a more effective agonist for the induction of [Ca\textsuperscript{2+}]. Similarly, P2Y5 activation by FPP resulted in a higher SRE reporter activity than by LPA, even though there was a greater total binding of LPA than FPP to the membrane fraction of P2Y5-overexpressing cells. Those observations could be a reflection of the difficulties in using standard receptor binding assays for lipid ligands; however, the possibility exists that liganded P2Y5 interacts with other accessory proteins such as GPCR kinase, β arrestin, or other 7TM receptors (which are either coupled or uncoupled to G proteins), causing an enhancement of downstream signaling pathways (8, 27, 57). Therefore, the physiological actions of LPA and FPP in the intestine might not be simply the result of an increase in binding of a particular ligand.

LPA induces migration of intestinal cells in culture through the activation of Go\textsubscript{i} (22, 60). Other PTX-sensitive receptors have been demonstrated to induce intestinal cell migration that is linked to a decrease cell adhesion. For example, the activation of the NPY receptor family [a Go\textsubscript{i2/13-coupled receptor family (43)] increases hBRIE 380i cell motility through a process that is linked to a decrease in cell adhesion by decreasing the adhesion molecule CD63, increasing matrix metalloproteinase 3 and through the action of small GTPase cdc42 (34). P2Y5 might activate similar downstream signal cascades to reduce epithelial cell adhesiveness. P2Y5 may also alter cell adhesion through RTK-mediated pathways since our results indicate that P2Y5 transactivates EGFR and EGFR activation in the intestine has been reported to modulate cell migration and adhesion (14).

The involvement of P2Y5 in the LPA-mediated decrease in intestinal cell adhesion could be important for maintaining intestinal epithelia integrity in vivo, for example by restitution or anoikis (18, 34, 60). Furthermore, the differential activation of P2Y5 by various forms of LPA and FPP could result in distinct Go\textsubscript{i} coupling, activation of downstream effector molecules (e.g., p38 MAPK, PI3K, PLC, and PKC), and signaling events. P2Y5 may play a significant role in the maintenance of mucosal homeostasis along the crypt-to-villus axis by acting as a means for the epithelium to respond to a range of extracellular cues, which can reflect the state of cell-to-cell integrity, cell metabolism, or the content of molecules in the lumen including those derived from the diet or intestinal flora.

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