P2Y₂ nucleotide receptor activation enhances the aggregation and self-organization of dispersed salivary epithelial cells

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El-Sayed FG, Camden JM, Woods LT, Khalafalla MG, Petris MJ, Erb L, Weisman GA. P2Y₂ nucleotide receptor activation enhances the aggregation and self-organization of dispersed salivary epithelial cells. Am J Physiol Cell Physiol 307: C83–C96, 2014. First published April 24, 2014; doi:10.1152/ajpcell.00380.2013.—Hyposalivation resulting from salivary gland dysfunction leads to poor oral health and greatly reduces the quality of life of patients. Current treatments for hyposalivation are limited. However, regenerative medicine to replace dysfunctional salivary glands represents a revolutionary approach. The ability of dispersed salivary epithelial cells or salivary gland–derived progenitor cells to self-organize into acinar-like spheres or branching structures that mimic the native tissue holds promise for cell-based reconstitution of a functional salivary gland. However, the mechanisms involved in salivary epithelial cell aggregation and tissue reconstitution are not fully understood. This study investigated the role of the P2Y₂ nucleotide receptor (P2Y₂R), a G protein-coupled receptor that is upregulated following salivary gland damage and disease, in salivary gland reconstitution. In vitro results with the rat parotid acinar Par-C10 cell line indicate that P2Y₂R activation with the selective agonist UTP enhances the self-organization of dispersed salivary epithelial cells into acinar-like spheres. Other results indicate that the P2Y₂R-mediated response is dependent on epidermal growth factor receptor activation via the metalloproteases ADAM10/ADAM17 or the α5β1 integrin/Cdc42 signaling pathway, which leads to activation of the MAPKs JNK and ERK1/2. Ex vivo data using primary submandibular gland cells from wild-type and P2Y₂R⁻/⁻ mice confirmed that UTP-induced migratory responses required for acinar cell self-organization are mediated by the P2Y₂R. Overall, this study suggests that the P2Y₂R is a promising target for salivary gland reconstitution and identifies the involvement of two novel components of the P2Y₂R signaling cascade in salivary epithelial cells, the α5β1 integrin and the Rho GTPase Cdc42.

salivary gland reconstitution; P2Y₂ nucleotide receptor; EGF receptor; α5β1 integrin; Cdc42 Rho GTPase; extracellular ATP

Salivary glands are exocrine glands composed of multiple secretory end pieces called acini, which secrete saliva into the oral cavity via a system of branched ductal cells, including intercalated ducts, striated ducts, and a main excretory duct (52). Saliva performs many protective and physiological functions by providing the oral cavity with water and electrolytes along with essential proteins, including lubricants, antibacterial, antifungal, antiviral, and remineralization agents, digestive enzymes, and growth factors (25, 31, 52). Accordingly, hyposalivation due to salivary gland dysfunction resulting from the autoimmune disease Sjögren’s syndrome (SS) or irradiation therapy for head and neck cancers leads to a significant deterioration of oral health and seriously decreases the quality of life of these patients (2, 4). Current treatments for hyposalivation are limited to saliva substitutes in the form of gels or sprays and medications, such as the muscarinic receptor agonists pilocarpine and cevimeline, which induce saliva secretion from residual salivary gland cells. However, these treatments are largely ineffective due to their transient nature or systemic side effects that are poorly tolerated by many patients (3, 106). Therefore, the development of new therapeutic approaches to treat salivary hypofunction is a necessity. Experimental approaches for restoring salivary gland function have been considered, including gene therapy to augment the expression of proteins involved in saliva secretion (28, 82, 104). An alternative approach to regain the function of salivary glands is to induce the proliferation, migration, and differentiation of residual cells in the damaged salivary glands to promote tissue regeneration (59). This approach can be further applied to bioengineer artificial salivary glands that closely resemble the native organ in both structure and function (59).

Reconstitution studies, using salivary gland tissue isolated from embryonic mice (118) and humans (90) or human salivary gland progenitor (SGP) cells (84), have demonstrated the ability of dissociated cells to migrate towards each other and self-organize into acinar-like aggregates with structural features and differentiation markers that resemble the native gland. Cellular mechanisms and components that enhance the formation of these acinar-like aggregates would likely be important factors in salivary gland reconstitution and regeneration. In this study, we investigated the role in salivary gland reconstitution/regeneration of the P2Y₂ nucleotide receptor (P2Y₂R) for extracellular ATP and UTP, since previous findings have suggested roles for the P2Y₂R in corneal epithelia wound healing by inducing cell migration (119), liver regeneration by promoting hepatocyte proliferation (8), inflammatory bowel disease by enhancing epithelial repair (27), intestinal reepithelialization following experimental colitis (26), and reduction of infarct size following myocardial infarction (20). In addition, the P2Y₂R is upregulated upon disruption of salivary gland tissue homeostasis (113) and in salivary glands of the NOD.B10 mouse model of SS-like autoimmune exocrinopathy (99). In a classic model of salivary gland regeneration, P2Y₂R expression and activity increase due to tissue atrophy caused by a 3-day ductal ligation in rat submandibular gland (SMG), whereas P2Y₂R expression and activity levels and glandular morphology resemble unligated controls 14 days after deligation (1). Collectively, these findings suggest that P2Y₂R upregulation plays a role in salivary gland regeneration.
The P2Y₂R has structural motifs that enable interactions with diverse signaling pathways, such as Src homology 3 binding domains that mediate transactivation of growth factor receptors (76) and an Arg-Gly-Asp (RGD) domain that binds directly to αvβ3 integrins to activate the Rho and Rac GTPases and cytoskeletal rearrangements (6, 34, 74). P2Y₂R activation also has been shown to induce downstream activation of MAPKs, including JNK (29, 110) and ERK1/2 (13, 29, 94), in several cell types. The P2Y₂R also has been shown to activate metalloproteases that induce epidermal growth factor (EGF) receptor (EGFR) and ErbB3 phosphorylation in salivary gland (HSG) cells (94). P2Y₂R interactions and signaling pathways enable extracellular ATP and UTP to regulate numerous physiological processes, such as cell proliferation, migration, and differentiation (6, 12, 14, 34, 87, 119, 125).

On the basis of the role of the P2Y₂R in the regulation of intracellular signaling pathways that are crucial to tissue repair, we investigated whether the P2Y₂R plays a similar role in the salivary gland using in vitro and ex vivo approaches. Our goals were to test whether P2Y₂R activation enhances the aggregation and self-organization of dispersed salivary epithelial cells into acinar-like aggregates and to determine the underlying mechanisms. We found that P2Y₂R-mediated formation of acinar-like spheres involves the transactivation of the EGFR through activation of the metalloproteases ADAM10/ADAM17 and the αvβ₃ integrin/Cdc42 Rho GTPase signaling pathway, leading to downstream activation of MAPKs.

In the following studies, we used the rat parotid acinar (Par-C10) cell line, an established in vitro model of salivary gland differentiation and function (7, 112). Par-C10 cells express endogenous P2Y₂Rs, but not P2Y₄Rs (unpublished observations) or P2Y₆Rs (112). Therefore, P2Y₂R is the only P2Y or P2X receptor subtype that responds to UTP in these cells (112). Unlike the majority of salivary cell lines, including HSG cells, Par-C10 cells are able to differentiate on Matrigel into three-dimensional (3D) acinar-like spheres that display characteristics similar to differentiated acini in salivary glands, including cell polarization and tight junction formation, which are required to maintain the transepithelial potential difference and responsiveness to muscarinic receptor agonists (7). In addition, we used primary SMG cells isolated from wild-type and P2Y₂R⁻/⁻ mice to corroborate the results obtained with Par-C10 cells.

MATERIALS AND METHODS

Reagents. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Par-C10 cell culture. Par-C10 cells transfected with cDNA encoding the green fluorescent protein (GFP)-tagged human P2Y₂R (GFP-hP2Y₂R) (7) were cultured in a 1:1 mixture of DMEM-Ham’s F-12 medium (Life Technologies, Grand Island, NY) supplemented with 5% CO₂ and 95% air. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Migration assay. Par-C10 single-cell suspensions in DMEM/Ham’s F-12 medium (1:1) containing 1% (vol/vol) FBS were seeded (2 × 10⁵ cells/well) on a 24-well plate coated with growth factor reduced (GFR-) Matrigel (BD Biosciences, San Jose, CA) and incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air for 4 h. Then, the cell culture plate was mounted on a Nikon Eclipse Ti-E microscope equipped with a digital camera, a motorized x-y stage, an automatic shutter, and an in vivo incubation chamber (37°C, 5% CO₂, and 95% air). Within each well, a field of cells was located with a ×10 objective and marked for monitoring over the duration of the experiment using Nikon NIS-Elements imaging software. The exposure time was kept constant for all positions and all time points. Cells were treated with or without UTP (100 µM) or EGF (100 ng/ml). In inhibitor studies, cells were pretreated with EGFR inhibitor AG1478 (1 µM) (Cell Signaling Technology, Beverly, MA), ADAM10/ADAM17 inhibitor TAPI-2 (10 µM) (PepTides International, Louisville, KY), αvβ₃ integrin blocking antibody (100 µg/ml) (Biolegend, San Diego, CA), Cdc42 inhibitor ML141 (10 µM) (Tocris Bioscience, Minneapolis, MN), RhoA inhibitor SR3677 (10 µM) (Tocris Bioscience), MEK/ERK pathway inhibitor U0126 (10 µM) (Cell Signaling Technology) or JNK inhibitor SP600125 (10 µM) (Tocris Bioscience) for 2 h before UTP, EGF, or vehicle (basal) treatment. Unless otherwise noted, inhibitors at the concentrations employed had no effect on the responses measured under basal conditions. Transmitted light images of cells were obtained every 10 min for the time indicated. Cellular aggregation was monitored by manually counting the number of aggregation events, where one aggregation event was defined as the coalescence/fusion of two or more cells at the same time point. ZO-1 tight junction protein was detected in Par-C10 cell aggregates formed after 36 h using immunofluorescence as previously described (7).

For primary SMG cells isolated from wild-type and P2Y₂R⁻/⁻ mice, SMGs were enzymatically dispersed and incubated for 3 days (37°C, 5% CO₂, and 95% air) to allow the P2Y₂R to upregulate. After 3 days, cells were serum-starved overnight and on day 4, cells were seeded on GFR-Matrigel for 8 h, treated with or without UTP, and monitored by time-lapse live cell imaging, as described above. In inhibitor studies, cells were pretreated with EGFR inhibitor AG1478 (1 µM) (Cell Signaling Technology) for 2 h before UTP or vehicle (basal) treatment. Primary cell migration was assessed by measuring the distance traveled from the origin, total distance traveled, and housed in vented cages with 12:12-h light-dark cycles and received food and water ad libitum. All animals were handled using protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Missouri, Columbia, MO. Animals were
average velocity using the tracking software provided with the NIS-Elements imaging software.

SDS-PAGE and Western blot analysis. Par-C10 cells (2 × 10^5 cells/well) were seeded on 24-well culture dishes, grown to 70% confluence, and then incubated overnight in DMEM-Ham’s F-12 medium (1:1) without serum. When indicated, the cells were pre-treated with or without inhibitors for 2 h at 37°C before stimulation with agonists for the indicated times. Then, the medium was removed and 100 μL of 2X Laemmli lysis buffer [20 mM NaH2PO4, pH 7.0, 20% (vol/vol) glycerol, 4% (wt/vol) SDS, 0.01% (wt/vol) bromophenol blue, and 100 mM dithiothreitol] were added. The samples were sonicated for 5 s with a Branson Sonifier 250 (microtip; output level, 5; duty cycle, 50%), heated at 95°C for 5 min, and subjected to SDS-PAGE on 7.5% (wt/vol) polyacrylamide gels. The proteins resolved on the gel were transferred to nitrocellulose membranes and blocked for 1 h with 5% (wt/vol) nonfat dry milk in Tris-buffered saline containing 0.1% (vol/vol) Tween 20 (TBST). The blots were incubated overnight at 4°C in blocking solution or TBST with the following rabbit polyclonal antibodies used at 1:1,000 dilutions: anti-phospho-EGFR (Tyr1068) (Cell Signaling Technology), anti-phospho-INK (Thr183/Tyr185) (Cell Signaling Technology), anti-phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling Technology) or anti-ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA) as a loading control. The membranes were washed three times with TBST and incubated with horseradish peroxidase-linked goat anti-rabbit IgG antibody (1:2,000 dilution; Santa Cruz Biotechnology) at room temperature for 1 h. The membranes were washed three times with TBST and incubated with enhanced chemiluminescence reagent, and the protein bands detected on X-ray film were quantified using a computer-driven scanner and Quantity One software (Bio-Rad, Hercules, CA). The intensities of phosphorylated protein bands in cells treated with agonists or other agents were normalized to total ERK1/2 and are expressed as a percentage of normalized data from untreated controls.

Cdc42 activation assay. A Cdc42 activation assay kit (Cell Biolabs, San Diego, CA) was used to assess Cdc42 activity according to the manufacturer’s instructions. Briefly, Par-C10 cells were cultured in 100-mm culture dishes and grown to 70% confluence. Then, the cells were starved overnight in serum-free DMEM-Ham’s F-12 medium (1:1) before being stimulated with UTP (100 μM) for the indicated times. Cell lysates were collected using 1X assay buffer (125 mM HEPES, pH 7.5, 750 mM NaCl, 5% NP-40, 50 mM MgCl2, 5 mM EDTA, 10% glycerol) and incubated for 1 h at 4°C with p21-activated kinase-1 p21-binding domain (PAK1 PBD) agarose beads, which bind the GTP-bound form of Cdc42. GTP-bound Cdc42 was analyzed by Western analysis using mouse monoclonal anti-rat Cdc42 antibody (1:1,000 dilution; Cell Biolabs). The membranes were washed three times with TBST and incubated with horseradish peroxidase-linked goat anti-mouse IgG antibody (1:2,000 dilution) at room temperature for 1 h. The membranes were washed three times with TBST and incubated with enhanced chemiluminescence reagent, and the protein bands were detected on X-ray film.

Reverse transcription and real-time PCR analysis of P2Y2R mRNA expression. Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA) from SMG aggregates cultured for 0, 24, 48, or 72 h at 37°C in 5% CO2 and 95% air. cDNA was synthesized from 1 μg of purified RNA using the Advantage RT for PCR kit (Clontech Laboratories, Mountain View, CA). Ten percent of the synthesized cDNA was used as a template in 25 μl real-time PCR reactions, and samples were run in duplicate for the P2Y2R target and the endogenous 18S RNA control. The relative levels of P2Y2R and 18S RNA in each sample were determined and are expressed as a ratio of P2Y2R to 18S RNA (normalized to 1) using Applied Biosystems software.

Intracellular free Ca2+ concentration measurements. Changes in the intracellular free Ca2+ concentration ((Ca2+)]i) in SMG cell aggregates were quantified as previously described (99). Briefly, dispersed SMG aggregates from wild-type or P2Y2R−/− mice were cultured for 72 h and loaded with 2 μM fura 2-AM (Calbiochem) for 30 min at 37°C in assay buffer (in mM: 120 NaCl, 4 KCl, 1.2 KH2PO4, 1.2 MgSO4, 1 CaCl2, 10 glucose, 15 HEPES, pH 7.4) containing 0.1% (vol/vol) BSA. Then, the SMG aggregates were washed and adhered to chambered coverslips coated with Cell-Tak (BD Biosciences) for an additional 30 min in the absence of fura 2-AM. SMG aggregates were stimulated with UTP (100 μM), and changes in the 340/380 nm excitation ratio (505 nm emission) were monitored using an InCyt dual-wavelength fluorescence imaging system (Intracellular Imaging, Cincinnati, OH). Fluorescence ratios were converted to [Ca2+]i (nM) using a standard curve created with known concentrations of Ca2+.

Statistical analysis. The quantitative results are presented as the means ± SE of data from three or more experiments. Two-tailed t-test or ANOVA followed by Bonferroni or Dunnett’s test was performed, as indicated, where P < 0.05 represents a significant difference.

RESULTS

P2Y2R activation enhances Par-C10 cell aggregation and the formation of acinar-like spheres. When plated on extracellular matrices, such as Matrigel, dispersed salivary epithelial cells isolated from embryonic mice (118) or adult humans (90) as well as cultured Par-C10 (7) and HSG (49) cells migrate towards each other and self-organize into aggregates that display structural and/or functional features similar to the native salivary gland. Since activation of the P2Y2R has been shown to enhance the migration of a variety of cell types (6, 117, 125), including epithelial cells (13, 68), we investigated whether P2Y2R activation enhances the migration, aggregation, and self-organization of salivary epithelial cells. Par-C10 single-cell suspensions seeded on GFR-Matrigel-coated 24-well plates (2 × 10^5 cells/well) were treated with or without UTP (100 μM), and cells were monitored for 36 h by time-lapse live cell imaging (Fig. 1A), as described in MATERIALS AND METHODS. During the first 2 h of the time course, UTP-treated single Par-C10 cells showed enhanced migratory responses, as indicated by the distance that single cells traveled from the origin (Fig. 1B), the total distance that cells migrated (Fig. 1C), and the increase in the cell velocity (Fig. 1D). After 2 h, single Par-C10 cells began to form aggregates that were quantified, as described in MATERIALS AND METHODS, where one aggregation event represents the coalescence/fusion of two or more cells at the same time point. UTP-treated Par-C10 cells exhibited enhanced aggregation (Fig. 1, A and E, and Supplemental Movies S1 and S2; Supplemental Material for this article is available online at the journal website) with 100% forming enhanced aggregation (Fig. 1, A and E, and Supplemental Movies S1 and S2; Supplemental Material for this article is available online at the journal website) with 100% forming acinar-like spheres that display lumen formation and an organized distribution of the tight junction protein ZO-1 (Fig. 1F) at the end of 36 h. Although untreated (basal) Par-C10 cells can aggregate and express ZO-1, they did not form differentiated acinar-like spheres until ~72 h in culture (data not shown), as previously described (7). Notably, the majority of aggregation events took place in the first 12 h after addition of UTP (Fig. 1G). Therefore, subsequent experiments investigating aggregation events were performed for 12 h.

Inhibition of EGFR decreases UTP-induced Par-C10 cell aggregation. EGFR regulates a wide variety of cellular responses, including cell migration and differentiation (47, 57). In HSG cells, the P2Y2R has been shown to activate EGFR (94). To determine whether UTP-induced enhancement of Par-C10 cell aggregation is dependent on EGFR activation, cells were pretreated with AG1478 (1 μM), a potent EGFR

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inhibitor, 2 h prior to UTP stimulation. The results show that EGFR inhibition decreased UTP-induced Par-C10 cell aggregation by 69% (Fig. 2A) and, as expected, completely inhibited EGF-induced enhancement of Par-C10 cell aggregation (Fig. 2A). EGFR inhibition also prevented UTP- and EGF-induced phosphorylation of the EGFR (Fig. 2B).

Inhibition of ADAM10/ADAM17 metalloproteases decreases UTP-induced Par-C10 cell aggregation and EGFR phosphorylation. In HSG cells, the P2Y<sub>2</sub>R has been shown to activate EGFR via ADAM10 and ADAM17 metalloproteases (94) that promote shedding of EGFR-like ligands, such as neuregulin, which bind to and activate members of the EGFR family. To determine whether ADAM10/ADAM17 are involved in the UTP-induced enhancement of Par-C10 cell aggregation, cells were pretreated with the selective ADAM10/ADAM17 inhibitor TAPI-2 (10 μM), which partially (52%) decreased UTP-induced Par-C10 cell aggregation (Fig. 3A), whereas ADAM10/ADAM17 inhibition did not affect the EGF-induced increase in aggregation (Fig. 3A). Consistent with the role of metalloproteases in the P2Y<sub>2</sub>R-mediated generation of EGFR agonists (94), ADAM10/ADAM17 inhibition decreased UTP-induced, but not EGF-induced, phosphorylation of the EGFR (Fig. 3B). The partial loss of UTP-induced Par-C10 cell aggregation by ADAM10/ADAM17 inhibition

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**Fig. 1.** UTP enhances Par-C10 cell aggregation and the formation of acinar-like spheres on growth factor reduced (GFR) Matrigel. **A:** Par-C10 single-cell suspensions were cultured on GFR-Matrigel for 4 h, then treated with or without UTP (100 μM) and monitored by time-lapse live cell imaging for 36 h, as described in MATERIALS AND METHODS. **B–D:** the migration of single Par-C10 cells was monitored for 2 h, and the distance migrated from the origin (B), total distance traveled (C), and average velocity of single cells (D) were quantified with the tracking software provided with NIS-Elements imaging software. The data represent the means ± SE of results from at least 3 experiments. *P < 0.05, significant increase over basal levels (two-tailed t-test). **E:** quantification of the number of aggregation events in response to UTP (100 μM) after 36 h. **F:** after 36 h, UTP-treated Par-C10 cell aggregates formed acinar-like spheres that display lumen formation and an organized distribution of the tight junction protein ZO-1 (red) detected by immunofluorescence using rabbit anti-ZO-1 antibody, as previously described (7), features not observed in the Par-C10 cell aggregates formed under basal conditions. **G:** quantification of the aggregation events from 0–12 h, 12–24 h, and 24–36 h indicates that the majority of aggregation events take place in the first 12 h with or without UTP treatment. The data shown represent the means ± SE of results from at least 3 experiments. *P < 0.05, significant increase over basal levels (two-tailed t-test).
suggested that other P2Y2R-mediated signaling pathways contribute to the cell aggregation response.

Inhibition of the α5β1 integrin/Cdc42 signaling pathway decreases UTP-induced Par-C10 cell aggregation and EGFR phosphorylation. The P2Y2R contains an extracellular-oriented RGD sequence that interacts with RGD-binding integrins (35) to activate Rho GTPases that regulate cell migration (6, 117). The RGD-binding α5β1 integrin has been shown to regulate SMG branching morphogenesis (96) and stimulate migration of a variety of cell types (16, 22, 42, 51, 69, 73, 115). To test whether UTP-induced Par-C10 cell migration and aggregation require activation of α5β1 integrin, cells were pretreated with α5β1 integrin function-blocking antibody (100 mg/ml) prior to addition of 100 μM UTP. Results indicate that inhibition of α5β1 integrin function decreased UTP-induced aggregation by 49%, but had no effect on the EGF-induced response (Fig. 4A). Integrin-dependent activation of the Rho GTPases Cdc42, Rac1, and RhoA has been shown to mediate cytoskeletal reorganization and cell migration, where there is a reciprocal relationship between Cdc42/Rac1 and RhoA activities (32, 75, 97). Our results show that stimulation of Par-C10 cells with 100 μM UTP activates Cdc42 in a time-dependent manner (Fig. 4B, top). Inhibition of Cdc42 with the selective antagonist ML141 (10 μM) significantly increased basal cell aggregation by almost twofold and had no additional effect on the UTP- or the EGF-induced response (two-tailed t-test). However, inhibition of Rac1 using NSC23766 (100 μM) did not affect UTP-induced or basal Par-C10 cell aggregation (data not shown). These data suggest that the P2Y2R-mediated activation of the α5β1 integrin/Cdc42 signaling pathway enhances the self-organization of Par-C10 cells into acinar-like spheres through the activation of the EGFR pathway. Moreover, these data show that RhoA plays an inhibitory role in the basal aggregation of dispersed Par-C10 cells.
UTP-induced Par-C10 cell aggregation depends on the activation of JNK and ERK1/2. The activation of the EGFR leads to downstream activation of the MAPKs ERK1/2 and JNK, a response shown to modulate cell migration (18). Initially, we determined that P2Y2R activation with 100 μM UTP stimulates the time-dependent phosphorylation of JNK and ERK1/2 in Par-C10 cells (Fig. 5A). Inhibition of JNK with 10 μM SP600125 or ERK1/2 with 10 μM U0126, a MEK inhibitor, decreased UTP-induced Par-C10 cell aggregation (Fig. 5B), suggesting that JNK and ERK1/2 are regulators of Par-C10 acinar-like sphere formation. The EGFR pathway is apparently involved in the regulation of JNK- and ERK1/2-dependent Par-C10 cell aggregation induced by 100 μM UTP, since inhibition of the EGFR with 1 μM AG1478 (Fig. 5C) significantly reduced UTP-induced JNK and ERK1/2 phosphorylation.

UTP stimulates the migration of primary murine SMG cells from wild-type but not P2Y2R−/− mice. To test whether the UTP-induced salivary epithelial cell migration and aggregation are mediated by the P2Y2R, SMGs from wild-type and P2Y2R−/− mice were isolated, enzymatically dispersed, and cultured for 3 days to allow for upregulation of the P2Y2R, as previously described (113). Similar to primary rat SMG cell aggregates (113), P2Y2R mRNA expression is upregulated with time in cells cultured from wild-type mice (Fig. 6A), consistent with an increase in the [Ca2+]i induced by UTP in these cells (Fig. 6B), responses not seen in SMG cell aggregates from P2Y2R−/− mice (data not shown). UTP (100 μM) stimulated the migration of SMG cell aggregates from wild-type but not P2Y2R−/− mice (Fig. 6C). UTP-induced migration of wild-type primary SMG cells occurred only during the first 4 h, as indicated by the distance that cell aggregates traveled from the origin (Fig. 6D), the total distance that cell aggregates migrated (Fig. 6E), and an increase in the velocity of the cell aggregates (Fig. 6F). A lack of these responses in SMG from P2Y2R−/− mice (Fig. 6, C–F) confirms that UTP-induced SMG cell migration is mediated by P2Y2R activation. Furthermore, inhibition of EGFR with 1 μM AG1478 impaired the UTP-induced migratory responses in wild-type primary SMG cells (Fig. 7), corroborating our findings using Par-C10 cells that the P2Y2R-induced responses are dependent on EGFR activation.
DISCUSSION

Recent progress has been made in the development of strategies for regeneration and engineering of a variety of tissues, including skin (79, 92), corneal epithelium (43), cartilage (85), bone (10, 11), bladder (77), and lacrimal (48) and salivary glands (83). Determining the capacity of dispersed salivary epithelial cells or salivary gland-derived progenitor cells to reassemble into acinar-like spheres or branching structures has been previously assessed (84, 90, 118), but little is known about the signaling events involved in these differentiation processes. In the present study, we determined that the P2Y2 nucleotide receptor (P2Y2R), known to be upregulated during salivary gland damage and disease (1, 99, 113), plays a role in the aggregation and self-organization of dispersed salivary epithelial cells into acinar-like spheres. Our in vitro data using the rat parotid acinar (Par-C10) cell line show that P2Y2R activation by UTP significantly enhances the migration, aggregation, and self-organization of dispersed Par-C10 cells into acinar-like spheres (Fig. 1, A–E, and Supple-
ADAM10 and ADAM17) and represent the means as the percentage increase in JNK (Thr183/Tyr185) and ERK1/2 (Thr202/Tyr204) phosphorylation induced by UTP, compared with untreated control, and ex vivo data show that gland (Fig. 1), as previously described (7). In addition, our ex differentiation markers similar to those of acini in the native mental Movies S1 and S2) that display structural features and characteristics of Par-C10 cells. A schematic outlining these P2Y2R-mediated signaling pathways involved in salivary epithelial cell migration and aggregation is shown in Fig. 8.

In this paper, we demonstrate that UTP-induced enhancement of dispersed salivary epithelial cell aggregation occurs by two distinct signaling pathways coupled to activation of the P2Y2R: 1) the activation of metalloproteases (i.e., ADAM10/ADAM17) and 2) the activation of the α5β1 integrin/Cdc42 Rho GTPase pathway, major signaling pathways that activate various physiological processes (5, 95, 101, 109, 116, 123, 128). Both of these signaling pathways activate EGFR, which leads to the downstream activation of JNK and ERK1/2 that we demonstrate increases UTP-induced aggregation of Par-C10 cells. A schematic outlining these P2Y2R-mediated signaling pathways involved in salivary epithelial cell migration and aggregation is shown in Fig. 8.

It is well-established that the EGFR and its signaling pathways are critical for stimulating cell migration and the regeneration of a variety of tissues (30, 37, 57, 58, 81, 91). In salivary tissue reconstitution studies, exogenous EGF has been shown to be crucial for the self-organization of dispersed salivary gland-derived progenitor cells into branching structures (84). Several studies have shown that P2Y2R activation enhances epithelial cell migration, thereby accelerating wound healing and tissue regeneration (9, 12–14, 26, 62, 68) in part due to transactivation of the EGFR (12, 14, 62, 68). Our group has previously shown that the P2Y2R mediates transactivation of the EGFR in HSG cells through metalloprotease-dependent neuregulin release (94). In the present study, we demonstrate that the ADAM10/ADAM17/EGFR signaling pathway is required for P2Y2R-mediated aggregation of salivary epithelial cells (Figs. 2, 3, and 8), suggesting P2Y2R as a potential therapeutic target for promoting salivary gland regeneration or the ex vivo bioengineering of salivary glands, which represent promising alternative approaches to replace the current ineffective therapies for hyposalivation resulting from SS or irradiation therapy for head and neck cancers.

In addition to metalloprotease-dependent activation of the EGFR, the P2Y2R can activate EGFR through the α5β1 integrin/Cdc42 signaling pathway (Fig. 4). Our group has previously shown that the P2Y2R contains an RGD motif in its first
extracellular loop that enables receptor interaction with RGD-binding $\alpha_\perp\beta_3/5$ integrins to stimulate cell migration (6, 26, 64, 117). However, P2Y$_2$R interactions with other RGD-binding integrins have not been previously reported. In this study, we show for the first time that the $\alpha_\perp\beta_3$ integrin, a known mediator of SMG branching morphogenesis (96), cell migration, and tissue regeneration (16, 40–42, 51, 66, 67, 69, 73, 78, 88, 115, 121, 127), also plays a role in P2Y$_2$R-mediated salivary epithelial cell aggregation (Fig. 4A). We have also shown that the P2Y$_2$R/$\alpha_{\perp}\beta_3$ integrin interaction leads to the activation of Rac (6), a Rho GTPase critical for regulating cell migration (39, 44–46, 93, 98), epithelial morphogenesis (44, 114), and salivary acinar formation (23). In this study, we found no evidence that Rac1 is required for P2Y$_2$R-mediated salivary epithelial cell aggregation (data not shown), but rather Cdc42, another Rho GTPase known to regulate cell migration (38, 39, 44–46, 93, 98) and tissue regeneration (89, 126), regulates the aggregatory response to P2Y$_2$R activation (Fig. 4B). Previous reports from our lab have linked P2Y$_2$R-mediated cell migration to the activation of the Rho GTPase RhoA, as well as Rac1 (6, 64, 74, 103). Interestingly, RhoA inhibition in Par-C10 salivary epithelial cells increased basal cell aggregation by almost twofold and had no additional effect on the UTP- or EGF-induced enhancement of cell aggregation (Fig. 4C), suggesting...
that RhoA GTPase is a negative regulator of migratory responses in these cells. A reciprocal relationship between Cdc42 and RhoA has recently been described for mammary epithelial acinar morphogenesis (32). In contrast, another study has shown that inhibition of RhoA does not affect acinus formation by HSG cells (23).

It is well-established that MAPKs, including JNK and ERK1/2, regulate cell proliferation, migration, and differentiation (15, 18, 21, 36, 63, 72, 86, 102, 120), processes important for salivary gland morphogenesis (60, 70) and regeneration of a wide variety of tissues (19, 24, 50, 53, 54, 56, 65, 71, 80, 100, 105, 107, 111, 122, 124). The P2Y2R-mediated activation of ERK1/2 has been reported in HSG cells (94), corneal epithelial cells (13), and human coronary artery endothelial cells (HCAEC) (29). However, the ability of the P2Y2R to activate JNK has only been reported for HCAEC (29) and primary rat hepatocytes (110). Our data indicate that the P2Y2R agonist UTP activates JNK and ERK1/2 (Fig. 5A) through the canonical EGFR pathway (Fig. 5C) to enhance salivary epithelial cell aggregation and self-organization (Fig. 5B).

Growth factor receptors and integrins represent major signaling pathways that interact at different levels to regulate...
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various physiological processes (33, 55). The present study indicates that the P2Y2R transactivates the EGFR through the αsβ1 integrin/Cdc42 signaling pathway as well as the activation of the metalloproteases ADAM10/ADAM17, enabling extracellular nucleotides to enhance the aggregation and self-organization of dispersed salivary epithelial cells into acinar-like spheres on GFRA-Matrigel by increasing the activities of the MAPKs JNK and ERK1/2. Further work is needed to investigate whether other P2Y2R signaling pathways are involved in acinar-like sphere formation, such as the activation of the MAPK p38 that has been reported to promote the regeneration of salivary glands (24), skeletal muscle (17), and sciatic nerve (61) and to regulate corneal epithelial wound healing (105). Other potential P2Y2R signaling pathways involved in salivary epithelial cell migration include the activation of phosphatidylinositol-3-kinase (PI3-K)/protein kinase B (Akt) (117) and the Gα signaling pathway (6). Understanding the signaling events responsible for the aggregation and self-organization of dispersed salivary epithelial cells into acinar-like spheres should provide insights into novel approaches for the bioengineering of salivary glands (83) and should lead to better regenerative/replacement strategies for salivary glands damaged in human autoimmune diseases or as an unintended side effect of radiation treatments for head and neck cancers.

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