P2Y<sub>2</sub> 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. P2Y<sub>2</sub> 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 due to 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 P2Y<sub>2</sub> nucleotide receptor (P2Y<sub>2</sub>R), 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 P2Y<sub>2</sub>R activation with the selective agonist UTP enhances the self-organization of dispersed salivary epithelial cells into acinar-like spheres. Other results indicate that the P2Y<sub>2</sub>R-mediated response is dependent on epidermal growth factor receptor activation via the metalloproteases ADAM10/ADAM17 or the α<sub>3</sub>β<sub>1</sub> 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 P2Y<sub>2</sub>R<sup>−/−</sup> mice confirmed that UTP-induced migratory responses required for acinar cell self-organization are mediated by the P2Y<sub>2</sub>R. Overall, this study suggests that the P2Y<sub>2</sub>R is a promising target for salivary gland reconstitution and identifies the involvement of two novel components of the P2Y<sub>2</sub>R signaling cascade in salivary epithelial cells, the α<sub>3</sub>β<sub>1</sub> integrin and the Rho GTPase Cdc42.

salivary gland reconstitution; P2Y<sub>2</sub> nucleotide receptor; EGF receptor; α<sub>3</sub>β<sub>1</sub> 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 P2Y<sub>2</sub> nucleotide receptor (P2Y<sub>2</sub>R) for extracellular ATP and UTP, since previous findings have suggested roles for the P2Y<sub>2</sub>R in corneal epithelial 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 P2Y<sub>2</sub>R 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, P2Y<sub>2</sub>R expression and activity increase due to tissue atrophy caused by a 3-day ductal ligation in rat submandibular gland (SMG), whereas P2Y<sub>2</sub>R expression and activity levels and glandular morphology resemble unligated controls 14 days after deligation (1). Collectively, these findings suggest that P2Y<sub>2</sub>R 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 αβ₃ 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, 117, 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 αβ₃ 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 P2Y₄R (unpublished observations) or P2Y₁R (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<sup>−/−</sup> 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 2.5% (vol/vol) fetal bovine serum (FBS) (Life Technologies), insulin (5 μg/ml), transferrin (5 μg/ml), selenite (5 ng/ml), retinoic acid (0.1 μM), EGF (80 ng/ml), triiodothyronine (2 nM), hydrocortisone (1.1 μM), glutamine (5 mM), gentamicin (50 μg/ml), cholaera toxin (8.4 ng/ml), and G418 (0.5 mg/ml) (Mediatech, Manassas, VA) and maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Cells were cultured 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, Minneapolis, MN), RhoA 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) and 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).

**Migration assay.** Par-C10 single-cell suspensions in DMEM/Ham’s F-12 medium (1:1) containing 0.1% (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), αβ₃ 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<sup>−/−</sup> 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...
and the protein bands were detected on X-ray film. SDS-PAGE and Western blot analysis. Par-C10 cells (2 × 10^6 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 2× Laemmli lysis buffer [20 mM Na₂HPO₄, 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-JNK (Thr183/Tyr185) (Cell Signaling Technology), anti-p21-activated kinase-1 p21-binding domain (PAK1 PBD) agarose (Santa Cruz Biotechnology), anti-Cdc42 antibody (1:1,000 dilution; Cell Biolabs). 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 presented as the fold changes normalized to 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.

Reverse transcription and real-time PCR analysis. 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 the relative levels of the synthesized cDNA was used as a template in 25 µl real-time PCR reactions, and the relative levels of the synthesized cDNA was used as a template in 25 µl real-time PCR reactions, and the relative levels of the synthesized cDNA was used as a template in 25 µl real-time PCR reactions, and the relative levels of the synthesized cDNA was used as a template in 25 µl real-time PCR reactions. Changes in 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% (wt/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 × 10⁵ 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 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
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 P2Y2R has been shown to activate EGFR via ADAM10 and ADAM17 metalloproteases (94) that promote shedding of EGF-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 P2Y2R-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...
scares that other \( \text{P2Y}_{2R} \)-mediated signaling pathways contribute to the cell aggregation response.

Inhibition of the \( \alpha_5 \beta_1 \) integrin/Cdc42 signaling pathway decreases UTP-induced Par-C10 cell aggregation and EGFR phosphorylation. The \( \text{P2Y}_{2R} \)R 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 \( \alpha_5 \beta_1 \) 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 \( \alpha_5 \beta_1 \) integrin, cells were pretreated with \( \alpha_5 \beta_1 \) integrin function-blocking antibody (100 mg/ml) prior to addition of 100 \( \mu \)M UTP. Results indicate that inhibition of \( \alpha_5 \beta_1 \) integrin function decreased UTP-induced aggregation by 49%, but had no effect on the EGFR-induced response (Fig. 4A). Integrin-dependent activation of the Rho GTPases Cdc42, Rac1, and RhoA has been shown to modulate 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 \( \mu \)M UTP activates Cdc42 in a time-dependent manner (Fig. 4B, top). Inhibition of Cdc42 with the selective antagonist ML141 (100 \( \mu \)M) did not affect UTP-induced aggregation by 90% (Fig. 4B, bottom) and inhibited the UTP-induced phosphorylation of EGFR to a similar extent (Fig. 4D), but it had no effect on EGFR-induced responses, which are independent of metalloproteases (Fig. 3) and \( \alpha_5 \beta_1 \) integrin/Cdc42 (Figs. 4, A, B, and D). However, inhibition of RhoA with SR3677 (10 \( \mu \)M) significantly increased basal cell aggregation by almost twofold and had no additional effect on the UTP- or the EGF-induced enhancement of Par-C10 cell aggregation (Fig. 4C). Inhibition of Rac1 using NSC23766 (100 \( \mu \)M) for 2 h, then treated with or without UTP (100 \( \mu \)M) or EGF (100 ng/ml). Five minutes after UTP or EGF addition, protein extracts were prepared from Par-C10 cell aggregates and EGFR phosphorylation (Y1068) was determined by Western analysis. Representative blots are shown (top). Quantification of protein levels in blots (bottom) was performed using Quantity One software, as described in MATERIALS AND METHODS. The data are expressed as the percentage increase in EGFR phosphorylation induced by UTP or EGF, compared with untreated controls, and represent the means ± SE of results from at least 3 experiments.

Fig. 2. Inhibition of epidermal growth factor (EGF) receptor (EGFR) activation decreases UTP-induced enhancement of Par-C10 cell aggregation. A: Par-C10 single-cell suspensions plated on GFR-Matrigel for 4 h were pretreated with or without the EGFR inhibitor AG1478 (1 \( \mu \)M) for 2 h, then incubated with or without UTP (100 \( \mu \)M) or EGF (100 ng/ml). Par-C10 cell aggregates were monitored by time-lapse live cell imaging for 12 h. The data are expressed as percentages of the maximal number of aggregation events induced by UTP or EGF in the absence of AG1478 and represent the means ± SE of results from at least 3 experiments. **P < 0.01, ***P < 0.001, significant difference from the UTP- or EGF-induced response (two-tailed t-test).
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<sup>−/−</sup> mice.** To test whether the UTP-induced salivary epithelial cell migration and aggregation are mediated by the P2Y2R, SMGs from wild-type and P2Y2R<sup>−/−</sup> 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 [Ca<sup>2+</sup>]<sup>i</sup> induced by UTP in these cells (Fig. 6B), responses not seen in SMG cell aggregates from P2Y2R<sup>−/−</sup> mice (data not shown). UTP (100 μM) stimulated the migration of SMG cell aggregates from wild-type but not P2Y2R<sup>−/−</sup> 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<sup>−/−</sup> mice (Fig. 6C–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.
or EGF, as indicated. We have 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 (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-
Fig. 5. UTP-induced enhancement of Par-C10 cell aggregation is dependent on the activation of JNK and ERK1/2 by the EGFR. A: Par-C10 cells were serum-starved overnight and treated with or without 100 μM UTP for 1, 5, 10, or 15 min. Protein extracts were subjected to SDS-PAGE, and p-JNK (Thr183/Tyr185), p-ERK1/2 (Thr202/Tyr204), and ERK1/2 (loading control) were detected by Western analysis. B: Par-C10 single-cell suspensions plated on GFR-Matrigel for 4 h were pretreated with or without the JNK inhibitor SP600125 (10 μM) or the MEK/ERK inhibitor U0126 (10 μM) for 2 h. Cells were then treated with or without UTP (100 μM), and cell aggregation was monitored by time-lapse live cell imaging for 12 h. The data are expressed as percentages of the maximal number of aggregation events induced by UTP and represent the means ± SE of results from at least 3 experiments. **p < 0.01, ***p < 0.001, significant difference from the UTP-induced response (two-tailed t-test). C: Par-C10 cells were pretreated for 2 h with or without the EGFR inhibitor AG1478 (1 μM) then treated with or without UTP (100 μM) for 5 min. Protein extracts were subjected to SDS-PAGE, and p-JNK (Thr183/Tyr185), p-ERK1/2 (Thr202/Tyr204), and ERK1/2 (loading control) were detected by Western analysis (representative blots are shown on the left, where a black line represents noncontiguous lanes from the same gel). Quantification of protein levels was performed (right), as described in MATERIALS AND METHODS. The data are expressed as the percentage increase in JNK (Thr183/Tyr185) and ERK1/2 (Thr202/Tyr204) phosphorylation induced by UTP, compared with untreated control, and represent the means ± SE of results from at least 3 experiments. *p < 0.05, **p < 0.01, significant difference from the UTP-induced response (two-tailed t-test).

mental Movies S1 and S2) that display structural features and differentiation markers similar to those of acini in the native gland (Fig. 1F), as previously described (7). In addition, our ex vivo data show that P2Y2R deletion prevents the UTP-induced migration of primary murine SMG cell aggregates (Fig. 6), demonstrating that UTP-induced migratory responses of salivary epithelial cells are primarily mediated by P2Y2R activation.

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 αβ1 integrin/Cdc42 Rho GTPase pathway, major signaling pathways that activate various physiological processes (5, 95, 101, 108, 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 ADAM10/ADAM17/EGFR 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 αβ3ß5 integrins to stimulate cell migration (6, 26, 64, 117). However, P2Y2R interactions with other RGD-binding integrins have not been previously reported. In this study, we show for the first time that the αβ3ß1 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 P2Y2R-mediated salivary epithelial cell aggregation (Fig. 4A). We also have shown that the P2Y2R/αV 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 P2Y2R-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 P2Y2R activation (Fig. 4B). Previous reports from our lab have linked P2Y2R-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 classical 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...

Fig. 8. Proposed mechanisms for P2Y2R-mediated enhancement of salivary epithelial cell aggregation and formation of acinar-like spheres. The P2Y2R enhances the aggregation of dispersed salivary epithelial cells into acinar-like spheres through the activation of the EGFR and subsequent downstream activation of JNK and ERK1/2. P2Y2R mediates EGFR activation through two distinct pathways: the first pathway involves P2Y2R-mediated activation of matrix metalloproteases (i.e., ADAM10/ADAM17), which cleave membrane-bound EGFR ligands (94) leading to the activation of the EGFR, and the second pathway involves P2Y2R-mediated activation of the Arg-Gly-Asp (RGD) binding α5β1 integrin, which leads to activation of the Rho GTPase Cdc42 that also activates the EGFR. RhoA activation has an inhibitory effect on the basal aggregation of dispersed Par-C10 salivary epithelial cells. P2Y2R, P2Y2 receptor; ADAM, a disintegrin and metalloproteinase; GFR, growth factor receptor; NRG, neuregulin; EGFR, epidermal growth factor receptor; RhoA, Ras homolog gene family member A; Cdc42, cell division control protein 42 homolog.

Enhancement of salivary epithelial cell migration and aggregation forming acinar-like spheres

RhoA Cdc42 MAPks JNK and ERK1/2

P2Y2R

ATP/UTP

RGD

α5β1 Integrin

Metalloproteases e.g., ADAM10/17

GFR Ligands e.g., NRG

GFRs e.g., EGFR

Fig. 7. P2Y2R-induced migration of primary SMG cell aggregates is dependent on EGFR. Primary SMGs isolated from wild-type and P2Y2R−/− mice were enzymatically dispersed and incubated for 3 days (37°C, 5% CO2, and 95% air) to enable upregulation of the P2Y2R. After 3 days, cells were serum-starved overnight, seeded on GFR-Matrigel for 8 h, and pretreated for 2 h with or without the EGFR inhibitor AG1478 (1 μM). Cells were then treated with or without UTP (100 μM). Cell aggregates of similar size were monitored by time-lapse live cell imaging. Quantification of the distance migrated from the origin (A), the total distance traveled (B), and the average velocity (C) of aggregates during the first 4 h of the time course was performed 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 decrease from basal levels (two-tailed t-test).
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various physiological processes (33, 55). The present study indicates that the P2Y2R transactivates the EGFR through the α2β1 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 structures on GFR-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 G5 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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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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