Nucleotide-regulated Calcium Signaling in Lung Fibroblasts and Epithelial Cells from Normal and P2Y$_2$ Receptor (−/−) Mice*

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To test for the role of the P2Y$_2$ receptor (P2Y$_2$-R) in the regulation of nucleotide-promoted Ca^{2+} signaling in the lung, we generated P2Y$_2$-R-deficient (P2Y$_2$-R(−/−)) mice and measured intracellular Ca$^{2+}_i$ responses (ΔCa$^{2+}_i$) to nucleotides in cultured lung fibroblasts and nasal and tracheal epithelial cells from wild type and P2Y$_2$-R(−/−) mice. In the wild type fibroblasts, the rank order of potencies for nucleotide-induced ΔCa$^{2+}_i$ was as follows: UTP ≥ ATP ≫ ADP > UDP. The responses induced by these agonists were completely absent in the P2Y$_2$-R(−/−) fibroblasts. Inositol phosphate responses paralleled those of ΔCa$^{2+}_i$ in both groups. ATP and UTP also induced Ca$^{2+}_i$ responses in wild type airway epithelial cells. In the P2Y$_2$-R(−/−) airway epithelial cells, UTP was ineffective. A small fraction (25%) of the ATP response persisted. Adenosine and α,β-methylene ATP were ineffective, and ATP responses were not affected by adenosine deaminase or by removal of extracellular Ca^{2+}, indicating that neither P1 nor P2X receptors mediated this residual ATP response. In contrast, 2-methylthio-ADP promoted a substantial Ca$^{2+}_i$ response in P2Y$_2$-R(−/−) cells, which was inhibited by the P2Y$_1$ receptor antagonist adenosine 3′-5′-diphosphate. These studies demonstrate that P2Y$_2$-R is the dominant purinoceptor in airway epithelial cells, which also express a P2Y$_1$ receptor, and that the P2Y$_2$-R is the sole purinergic receptor subtype mediating nucleotide-induced inositol lipid hydrolysis and Ca^{2+} mobilization in mouse lung fibroblasts.

Extracellular ATP induces a wide variety of responses in many cell types, including muscle contraction and relaxation, vasodilation, neurotransmission, platelet aggregation, ion transport regulation, and cell growth (1–3). The cell surface receptors mediating these diverse effects of ATP were originally termed P2 purinoceptors to distinguish them from the adenosine-activated P1 purinoceptors (4). Subsequently, pyrimidine nucleotides were also shown to regulate a broad range of cell functions, leading to speculation about the existence of separate pyrimidoceptors (5, 6). It is likely, however, that a common receptor for uridine and adenine nucleotides is present

in many cell types, including neutrophils, pituitary cells, skin fibroblasts, smooth muscle cells, and specific endothelial and epithelial cell types (2). This receptor was originally named the P2U purinoceptor but has been subsequently reclassified as the P2Y$_2$ receptor (P2Y$_2$-R).[1] The cloning of the murine *P2Y$_2$-R* gene (7) and its human counterpart (8) made possible the definitive identification of this signaling protein as a G-protein and phospholipase C-coupled receptor that is equipotently activated by ATP and UTP but not by diphosphate nucleotides (9–11).

The lack of specific agonists or antagonists for the growing number of nucleotide receptor subtypes (*e.g.* seven P2X and five P2Y receptors have been identified to date (12, 13)) constitutes a major obstacle in identifying the specific nucleotide receptor mediating a given cellular function. One example of the difficulty in assigning receptor subtypes to cellular responses is illustrated in studies of fibroblasts. Following original studies by Okada *et al.* (14), who observed that ATP induced change in the membrane potential of mouse L cells and human fibroblasts, a variety of adenosine- and ATP-induced responses in fibroblasts were reported. These actions of adenosine and ATP, which include regulation of cell growth, cytoskeletal contraction, Ca^{2+} efflux, and LDH and nucleotide release (15–19), were proposed to be mediated by A$_1$, A$_2$, P2X, P2Z (in current terminology P2X$_7$), and P2Y$_1$ receptors (15–23). In one study with human skin fibroblasts, actions of ATP on Ca^{2+} mobilization and phospholipase C activity were mimicked by UTP (24), although no further characterization of the receptor(s) mediating UTP responses in fibroblasts was provided.

The effects of extracellular nucleotides have also been extensively studied on airway epithelia, and attempts have been made to link the cellular responses to specific nucleotide receptors. Both ATP and UTP equipotently regulate epithelial electrolyte and water transport (3, 25), trigger mucin secretion (26, 27), and increase ciliary beat frequency (28–30). ATP and UTP equipotently stimulate inositol phosphate formation (29) and Ca$^{2+}_i$ mobilization and exhibit cross-desensitization (3). These data suggest that a common receptor for ATP and UTP is expressed on the airway epithelia, which pharmacologically is most likely to be the P2Y$_2$-R. However, receptors that are activated by UDP (31) and adenosine (32) may also be expressed on these cells and thus complicate this analysis.

In this study, we generated a mouse line carrying a mutant *P2Y$_2$* allele. We used these mice to examine the relative role of P2Y$_2$-R in the nucleotide-promoted Ca^{2+} signaling in mouse

[1] The abbreviations used are: P2Y$_2$-R, P2Y$_2$ receptor; 2-MeSATP, 2-methylthioadenosine 5′-triphosphate; 2-MeSADP, 2-methylthioadenosine 5′-diphosphate; α,β-meATP, α,β-methylene adenosine 5′-triphosphate; A3P5P, adenosine 3′,5′-diphosphate; EC$_{50}$, 50% of maximal effective concentration; SSC, sodium chloride-sodium citrate.

lung fibroblasts and airway epithelial cells. The role of $P2Y_2$-R was tested by comparison of nucleotide-stimulated Ca^{2+}_i responses in cells from $P2Y_2$-R (−/−) mice with those from wild type animals. In the accompanying paper (33), the role of $P2Y_2$-R in mediating Cl^- secretory responses in freshly excised tracheal, gallbladder, and jejunal tissues is described.

MATERIALS AND METHODS

Generation of $P2Y_2$-R-deficient Mice—A targeting vector was designed such that DNA corresponding to base pairs 552–1149 of the published $P2Y_2$-R cDNA was replaced with the neomycin gene upon integration of the targeting plasmid into the genome by homologous recombination. The targeting plasmid contains two regions of DNA with homology to the endogenous locus. The targeting vector was constructed by cloning two genomic DNA fragments into the JNS2 vector: a 2500-base pair fragment extending from an *Xho*I site in the 5′ region of the gene to a *Sma*I site located at base pair 552 of the published cDNA and a fragment extending 6500 bases 3′ from the *Eag*I site at base pair 1149 of the coding sequence. The targeting vector was electroporated into E142aTG cells, and resulting neomycin- and gancyclovir-resistant colonies were isolated. DNA from surviving colonies was isolated, digested with *Bam*HI, and analyzed by Southern blot analysis using a probe located immediately upstream of the $P2Y_2$-R genomic fragments not included in the targeting vector. Chimeric mice were generated with $P2Y_2$-R-targeted E142aTG cell lines and were bred to B6D2 mice. Offspring were identified by Southern blot analysis of tail DNA, using probes described above.

Total cellular RNA was isolated from kidneys of $P2Y_2$-R(+/+) and $P2Y_2$-R(−/−) mice with RNAzol B (TelTest, Inc., Friendswood, TX), as per the manufacturer's instructions. Twenty μg of RNA were electrophoresed in a 1.2% agarose formaldehyde gel, blotted to an Immobilon-NC transfer membrane (Millipore Corp., Bedford, MA), and UV-cross-linked. The membranes were hybridized with a ^{32}P-labeled $P2Y_2$-R cDNA for 1 h at 68 °C using Quikhyb reagent (Stratagene, La Jolla, CA), and blots were washed twice with 2× SSC, 0.1% SDS and once with 0.2× SSC, 0.1%SDS at 42 °C for 15 min each. The $P2Y_2$-R cDNA corresponds to base pairs 267–1097.

For histological analysis, all animals were exsanguinated by severing the aorta after receiving an intraperitoneal injection of a lethal dose of chloral hydrate (1 ml of a 20 mg/ml solution). Organs were immersed in 10% phosphate-buffered neutral formalin (pH 7.0) for at least 24 h. The organs then were embedded in paraffin, dehydrated, and sectioned for histological analysis with hematoxylin and eosin.

Adult mice (wild type and $P2Y_2$(−/−)) of both sexes were used in this investigation. All animals were bred and raised at the University of North Carolina at Chapel Hill. All mice were allowed food and water *ad libitum* until euthanized.

Cell Culture—Wild type and $P2Y_2$-R(−/−) mice were euthanized with 100% CO_2. Lung fibroblasts were isolated by mincing freshly excised lung parenchyma into ~1-mm³ pieces and establishing explant cultures on plastic tissue culture plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Outgrowth fibroblasts were harvested with 0.1% trypsin plus 1 mM EDTA in phosphate-buffered saline 1–3 weeks after initial plating. The cells were seeded on glass coverslips coated with 0.3 mg/ml Vitrogen (Collagen Biomaterials, CA) and cultured for 36–48 h. Nearly confluent cultures were used for study.

To isolate epithelial cells, the trachea and nasal turbinates were removed from the animals and dissected free of blood vessels and connective tissues. The airway epithelial cells were disaggregated from the tissues by a 4-h treatment with 0.1% protease XIV (Sigma), epithelial cells isolated by centrifugation, and cells were seeded at a 5×10^5 cells/cm² density on Vitrogen-coated glass coverslips. The cells were allowed to attach for 24 h in Ham's F-12-based medium containing 10 μg/ml insulin, 5 μg/ml transferrin, 1 μM hydrocortisone, 30 nM triiodothyronine, 25 ng/ml epidermal growth factor, 3.75 μg/ml endothelial cell growth substance, 0.8 mM Ca^{2+} (total), and an equal amount of 3T3 fibroblast-conditioned Dulbecco's modified Eagle medium containing 2% fetal bovine serum, following which the cultures were gently washed and maintained for an additional 24–36 h before study. Only well attached cell clusters containing equal numbers of ciliated and nonciliated cells were used for Ca^{2+}_i studies.

Ca^{2+}_i Measurements—The cell cultures were washed with hormone-free Ham's F-12 medium and incubated with 3 μM Fura-2/AM for 30 min at 37 °C. After the loading period, the cells were washed twice with Ringer solution (130 mM Na^+, 128 mM Cl^-, 5 mM K^+, 1.3 mM Ca^{2+}, 1.3 mM Mg^{2+}, 5 mM glucose, and 10 mM Hepes, pH 7.4) and mounted in a
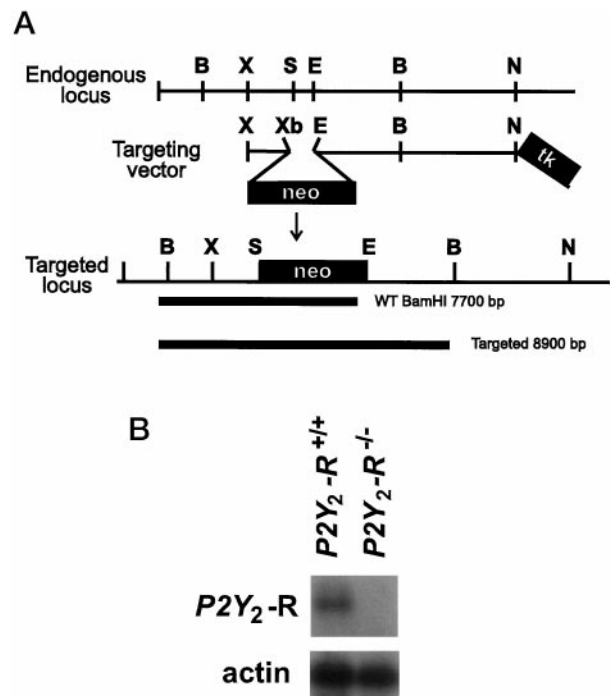

FIG. 1. **Targeted disruption of the $P2Y_2$-R gene.** *A*, construction of the $P2Y_2$-R targeting vector. *B*, confirmation of targeting by Northern blot analysis. RNA was generated from kidneys of a $P2Y_2$-R(+/+) and a $P2Y_2$-R(−/−) mouse. $P2Y_2$-R is present in the kidneys of the wild type mouse but is absent in the kidneys of the $P2Y_2$-R(−/−) mouse. *B, Bam*H1; *E, Eco*RI; *S, Sma*I; *X, Xba*I; *N, Not*I.

microscope chamber. The fluorescence (>450 nm) of 30–50 cells was alternately acquired at 340- and 380-nm excitation by a RatioMaster RM-D microscope fluorimetry system (Photon Technology Inc., Monmouth Junction, NJ) at room temperature. A Zeiss Axiovert 35 microscope and a Nikon UV-F × 100 (1.3) glycerol immersion objective were used. After each experiment, the cells were lysed with 40 μM digitonin, and the background fluorescence was determined by quenching technique using 4 mM $MnCl_2$.

Inositol Phosphate Studies—Inositol phosphate measurements were performed as described previously (34). In brief, cells grown on Vitrogen-coated glass coverslips were labeled overnight with 5 μCi/ml *myo*-[^3H]inositol in inositol-free Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose. The cells were then preincubated with 10 mM LiCl for 15 min and challenged with agonist for an additional 15 min. The incubations were terminated by the addition of 5% ice-cold trichloroacetic acid. The accumulated [^3H]inositol phosphates were separated on Dowex AG1-X8 anion exchange columns and quantified in a scintillation counter (31).

Reagents—Hormones for cell culture were purchased from Collaborative Research, Inc. (Bedford, MA) with the exception of triiodothyronine, which was from Sigma; other cell culture materials were purchased from Life Technologies, Inc. Molecular biology grade ATP and UTP were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Hexokinase, UDP, ADP, and adenosine were from Roche Molecular Biochemicals. 2-methylthio-ATP (2-MeSATP), 2-methylthio-ADP (2-MeSADP), α,β-methylene ATP (α,β-meATP), and digitonin were obtained from RBI (Natick, MA). Fura-2/AM, Fura-2 pentapotassium salt, and Ca^{2+} calibration buffers were purchased from Molecular Probes, Inc. (Eugene, OR). *myo*-[^3H]inositol (20 Ci/mmol) was from ARC (St. Louis, MO). All other chemicals were purchased from Sigma. To remove triphosphate contamination from diphosphate nucleotides, 1 mM stock solutions of UDP, ADP, and 2-MeSADP were pretreated with 10 units/ml hexokinase for 30 min at 37 °C in the presence of 5 mM glucose (10).

Data Analysis—For Ca^{2+}_i measurements, the background corrected ratio values (340/380) were calibrated by using the formula originally proposed by Grynkiewicz *et al.* (35). The optical parameters of the system, R_{max}, R_{min}, and K_d values were determined by using 1 μM Fura-2 free acid and a series of Ca^{2+} buffers. Differences between the peak and basal Ca^{2+}_i concentration were plotted. The data are presented as mean ± S.E. For comparisons, the mean values were analyzed
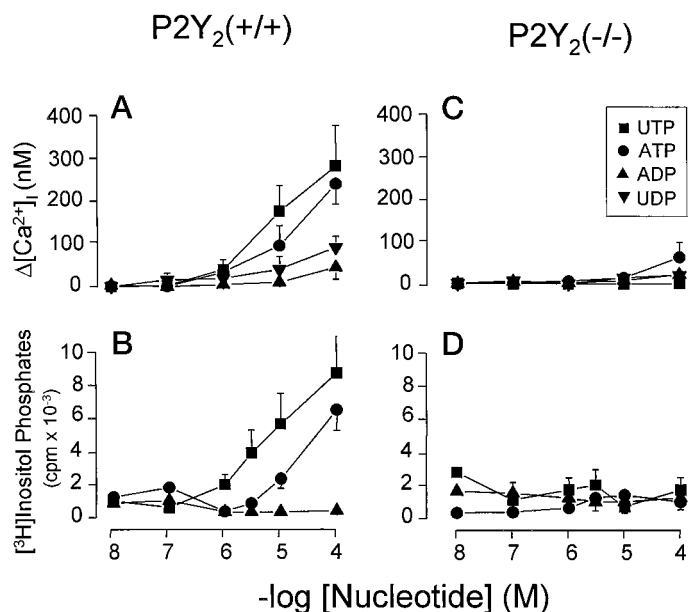
P2Y$_2$(+/+)   P2Y$_2$(-/-)

FIG. 2. **Concentration-effect curves for nucleotide-stimulated Ca^{2+} responses (ΔCa^{2+}$_i$) and [^{3}H]inositol phosphate formation in mouse lung fibroblasts.** Cells isolated from wild type (*A, B*) and P2Y$_2$-R(−/−) mice (*C, D*) were exposed to the indicated concentration of ATP (●), UTP (■), ADP (▼), or UDP (▲). Changes in Ca^{2+}$_i$ were measured immediately after the addition of agonist to Fura-2-loaded cells (*top panels*). Values are mean ± S.E. of changes from basal to peak concentration (*n* = 3–12/concentration). *myo*-[^{3}H]Inositol-labeled cells were preincubated with LiCl and subsequently challenged with the indicated nucleotide for 15 min (*B, D*). Counts from accumulated [^{3}H]inositol phosphates over the background were plotted. Each data point represents the mean ± S.E. of three independent experiments performed in triplicate.

by unpaired *t* tests. The significant differences (*p* < 0.05) are indicated by asterisks.

<center>RESULTS</center>

Generation of P2Y$_2$-R-deficient Mice—Mice deficient in P2Y$_2$–R were generated by targeted mutagenesis of the *P2Y$_2$-R* gene in mouse embryonic stem cells (Fig. 1*A*). RNA isolated from kidneys of a P2Y$_2$-R(+/+) and P2Y$_2$-R(−/−) mouse confirmed the complete loss of P2Y$_2$-R in the P$_2$Y$_2$ (−/−) mouse (Fig. 1*B*). Mice homozygous for the mutant P2Y$_2$-R allele were obtained at the expected frequency, were fertile, and could not be distinguished from wild type littermates. No differences were seen on histological analysis of all organs analyzed, including the kidney, heart, testes, pancreas, liver, trachea, lungs, salivary glands, and gastrointestinal tract.

Effects of Nucleotides on Inositol Phosphate Accumulation and Intracellular Ca^{2+} Levels in Murine Lung Fibroblasts— The effects of nucleotides were studied in cultured lung fibroblasts isolated from wild type and P2Y$_2$-R(−/−) mice. Changes in the intracellular Ca^{2+} concentration ([Ca^{2+}]$_i$) were monitored by using Fura-2 fluorescent indicator, and nucleotide-induced [^{3}H]inositol phosphate formation was measured in *myo*-[^{3}H]inositol-labeled cells (Fig. 2). In wild type fibroblasts, UTP and ATP promoted dose-dependent Ca^{2+}$_i$ (Fig. 2*A*) and inositol phosphate responses (Fig. 2*B*). ADP induced only a small Ca^{2+}$_i$ response at high concentrations. UDP had no substantial effect.

Both Ca^{2+}$_i$ and inositol phosphate responses to nucleotides were abolished in P2Y$_2$-R(−/−) fibroblasts (Fig. 2, *C* and *D*). Similarly, ADP and UDP did not induce responses over background in these cells. These data indicate that the P2Y$_2$ receptor is the only nucleotide receptor functionally expressed in murine lung fibroblasts.
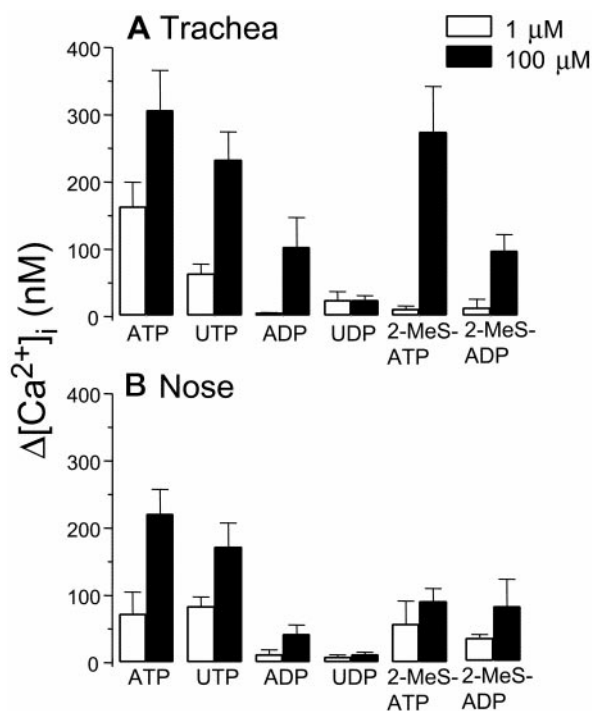

FIG. 3. **Nucleotide-induced Ca^{2+} responses (ΔCa^{2+}$_i$) in wild-type murine tracheal (*A*) and nasal (*B*) epithelial cells.** Changes in [Ca^{2+}]$_i$ in response to 1 and 100 μM concentrations of the indicated nucleotide were measured in Fura-2-loaded airway epithelial cells isolated from wild type mice. Maximal changes in [Ca^{2+}]$_i$ (from basal to peak) in response to the agonists were plotted (*n* = 3–14).

Characterization of Nucleotide-induced Responses in Wild Type Airway Epithelial Cells—Primary murine airway epithelial cells have a limited growth capacity. Therefore, in experiments with airway epithelia, we focused only on Ca^{2+}$_i$ measurements and confined our pharmacologic characterizations to two concentrations of nucleotide agonists. The two concentrations (1 and 100 μM) of nucleotides studied were selected on the basis of previous studies of human nasal cells, where 100 μM ATP and UTP induced a maximal effect, and their EC$_{50}$ values were in the low micromolar concentration range (3).

ATP and UTP promoted substantial Ca^{2+}$_i$ responses at both 1 and 100 μM concentrations in tracheal cells from wild type mice (Fig. 3*A*). ADP, 2-MeSATP, and 2-MeSADP were effective only at the 100 μM concentration, while UDP had no measurable effect. A similar pattern was found in nasal cells (Fig. 3*B*), with the exception that, in the latter, the maximal responses to 100 μM agonist concentrations were generally smaller, whereas the 2-MeSATP and 2-MeSADP responses were relatively larger at the 1 μM concentration.

To investigate possible cross-desensitization between agonists in nucleotide-promoted Ca^{2+}$_i$ responses, isolated tracheal and nasal cells were exposed first to successive additions of a 100 μM concentration of a given agonist until no further change in Ca^{2+}$_i$ signal was observed. Subsequently, the cells were exposed to 100 μM of a second agonist in the continued presence of the first one.

The results obtained from wild type tracheal cells are shown in Fig. 4. UTP pretreatment markedly, but not completely, reduced the Ca^{2+}$_i$ response to ATP (Fig. 4*A*), whereas ATP pretreatment completely abolished the UTP-induced Ca^{2+}$_i$ response (Fig. 4*B*). Pretreatment with ADP, and 2-MeSATP had no significant effect on ATP or UTP-induced Ca^{2+}$_i$ responses (Fig. 4, *A* and *B*). The Ca^{2+}$_i$ signal elicited by 2-MeSATP was entirely abolished by ATP pretreatment (Fig. 4*C*). UTP or 2-MeSADP pretreatment also reduced, although only partially,
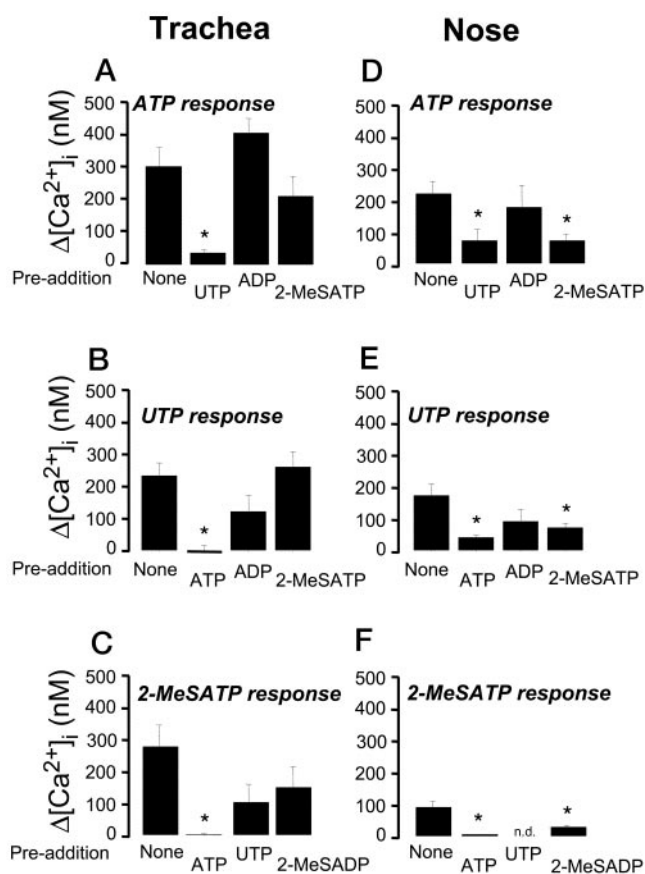
FIG. 4. **Desensitization of nucleotide-stimulated Ca^{2+} responses in wild type tracheal and nasal epithelial cells.** *A*, *B*, and *C*, tracheal Ca^{2+} responses induced by 100 μM ATP, UTP, or 2-MeSATP were measured following no preaddition (*None*) or preaddition of a 100 μM concentration of the nucleotides indicated at the *bottom* of each co*l*umn. Responsiveness of the cells was tested by adding 200 μM carbachol after each individual experiment. The values are mean ± S.E. (n = 3–7). *D*, *E*, and *F*, desensitization studies with wild type nasal epithelial cells were performed with the same protocol detailed above. The values are mean ± S.E. (n = 3–6). The *asterisks* indicate significant differences between responses with or without pretreatment ($p < 0.05$). *n.d.*, not determined)

the 2-MeSATP-induced responses. These findings suggest the functional expression of both a common receptor for ATP and UTP and an additional adenine nucleotide receptor(s).

Fig. 4 also summarizes the desensitization experiments performed with wild type nasal epithelial cells. A partial cross-desensitization between ATP and UTP was observed in this cell type (Fig. 4, *D* and *E*). Pretreatment with ADP did not significantly alter the response induced by ATP or UTP, whereas 2-MeSATP pretreatment significantly attenuated both ATP- and UTP-stimulated signals. The $Ca^{2+}{}_i$ response to 2-MeSATP was eliminated by ATP or 2-MeSADP pretreatment (Fig. 4*F*). Taken together, these results are also consistent with the expression of a common receptor for ATP and UTP and, possibly, an additional ADP receptor.

Nucleotide-induced Ca^{2+} Responses in Airway Epithelial Cells from $P2Y_2$-$R(-/-)$ Mice—A potential candidate for the common ATP/UTP receptor in airway epithelial cells is the $P2Y_2$ receptor. To test the involvement of $P2Y_2$ receptor in the murine airway epithelium, $Ca^{2+}{}_i$ studies were performed on tracheal and nasal cells isolated from $P2Y_2$-$R(-/-)$ mice. The UTP-induced $Ca^{2+}{}_i$ responses were abolished in both tracheal (Fig. 5*A*) and nasal cells (Fig. 5*B*). The magnitude of ATP-stimulated $Ca^{2+}{}_i$ responses was substantially reduced in $P2Y_2$-$R(-/-)$ tracheal and nasal epithelial cells, but residual $Ca^{2+}{}_i$

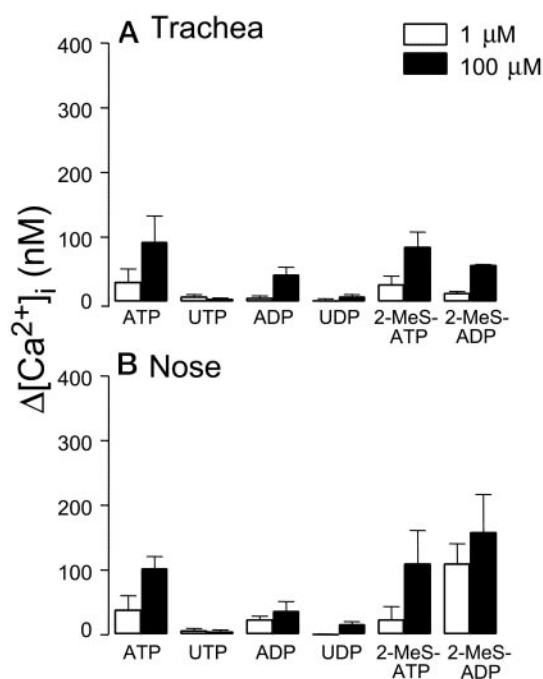FIG. 5. **Effect of nucleotides on tracheal (*A*) and nasal (*B*) epithelial cells isolated from $P2Y_2$-$R(-/-)$ mice.** For details, see the legend of Fig. 2.

responses were measurable. The reductions in the responses to 100 μM ATP in wild type and $P2Y_2$-$R(-/-)$ tracheal and nasal cells (Figs. 3 and 5, respectively) were 74.5 and 44.0% ($p < 0.02$), respectively.

ADP, 2-MeSATP, and 2-MeSADP elicited $Ca^{2+}{}_i$ responses in both tracheal and nasal cells from $P2Y_2$-$R(-/-)$ mice, whereas UDP had no substantial effect in either. A slight reduction in responses induced by 100 μM ADP and 2-MeSATP was observed in the $P2Y_2$-$R(-/-)$ tracheal cells, but $Ca^{2+}{}_i$ responses induced by 100 μM 2-MeSADP in both $P2Y_2$-$R(-/-)$ tracheal and nasal cells were not significantly different from that found in wild type cells.

These results clearly demonstrate that the $P2Y_2$ receptor is the major but not the unique nucleotide receptor functionally expressed in murine tracheal and nasal epithelial cells.

Identification of the Residual Nucleotide Receptor in $P2Y_2$-$R(-/-)$ Airway Epithelial Cells—Next, we initiated a series of experiments to identify the nucleotide receptor type(s) that accounted for the residual $Ca^{2+}{}_i$ responses induced by adenine nucleotides. To test for the involvement of adenosine receptors in ATP-promoted responses, $P2Y_2$-$R(-/-)$ cells were exposed to 100 μM ATP in the presence or absence of 1 unit/ml adenosine deaminase. In tracheal cells, ATP stimulated a 65.4 ± 17 nM (n = 3) change in $Ca^{2+}{}_i$ in the presence of enzyme, which was not significantly different from the values obtained in its absence, 92.4 ± 40 nM (n = 10). In nasal cells, the ATP-induced Ca^{2+} responses were 103.8 ± 65 (n = 4) and 108.9 ± 29 nM (n = 11) in the presence and absence of adenosine deaminase, respectively. In addition, no $Ca^{2+}{}_i$ response was elicited by 100 μM adenosine in either tracheal or nasal epithelial cells (1.5 ± 1.0 and 4.2 ± 0.2 nM, respectively (n = 3)).

Next, we tested for the possible involvement of P2X receptors (36). To investigate this issue, 100 μM α,β-meATP was applied to the $P2Y_2$-$R(-/-)$ tracheal and nasal cells. No $Ca^{2+}{}_i$ response was elicited by this compound in either cell type ($\Delta Ca^{2+}{}_i$ in nose, 5.2 ± 3.9 nM, n = 3; $\Delta Ca^{2+}{}_i$ in trachea, 4.8 ± 1.0 nM, n = 5). Further, ATP-induced $Ca^{2+}{}_i$ responses in Ca^{2+}-free buffer were not different from those found in the presence of 1.3 mM Ca^{2+} in either wild type (Fig. 6*A*) or $P2Y_2$-$R(-/-)$ (Fig. 6*B*)
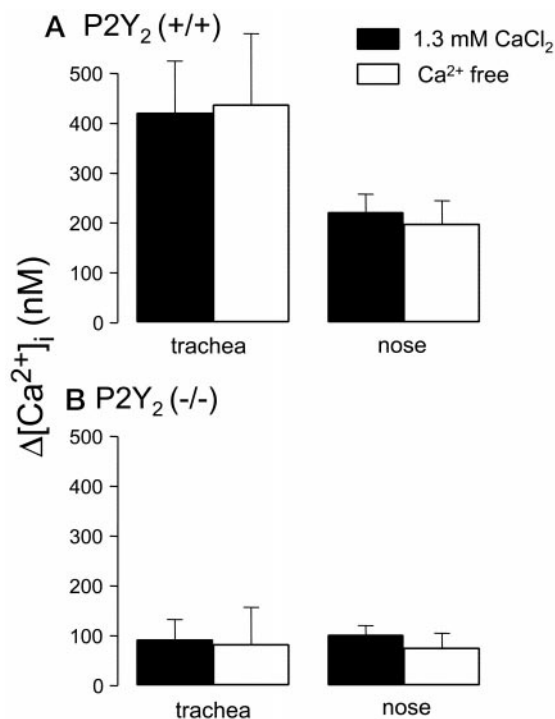
FIG. 6. **Effect of extracellular Ca²⁺ on the ATP-induced Ca²⁺ response in tracheal and nasal epithelial cells.** Cells isolated from wild type (*A*) and P2Y₂-R(−/−) (*B*) mice were challenged with 100 μM ATP in the presence and absence of extracellular Ca²⁺ (n = 3–11).
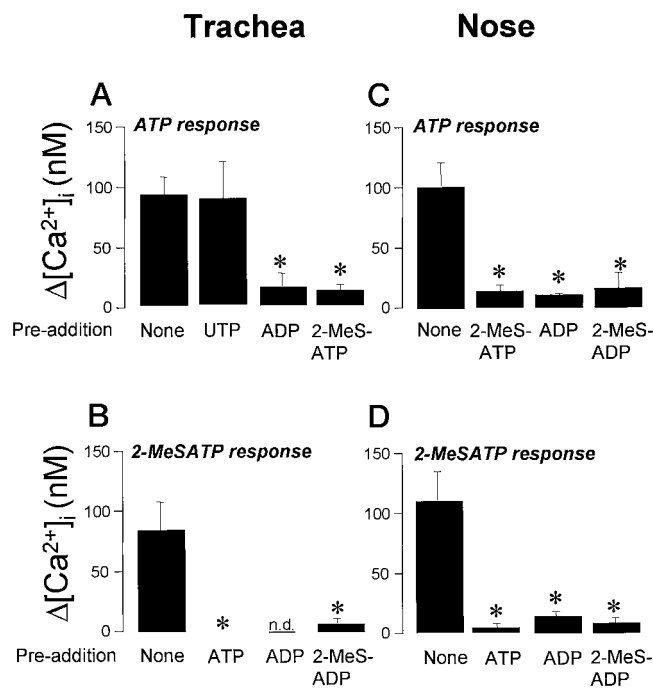

FIG. 7. **Desensitization studies in P2Y₂-R(−/−) tracheal and nasal epithelial cells.** *A*, tracheal cell Ca²⁺ responses induced by 100 μM ATP (*A*) or 2-MeSATP (*B*) following no preaddition (*None*), or preaddition of 100 μM of nucleotide indicated at the *bottom* of each *column*. Similar protocols were used with nasal epithelial cells (*C*, *D*). The experiments were carried out as described in the legend of Fig. 3. The values are mean ± S.E. (n = 3–7). The *asterisks* indicate significant differences between responses with or without pretreatment ($p < 0.05$). *n.d.*, not determined.
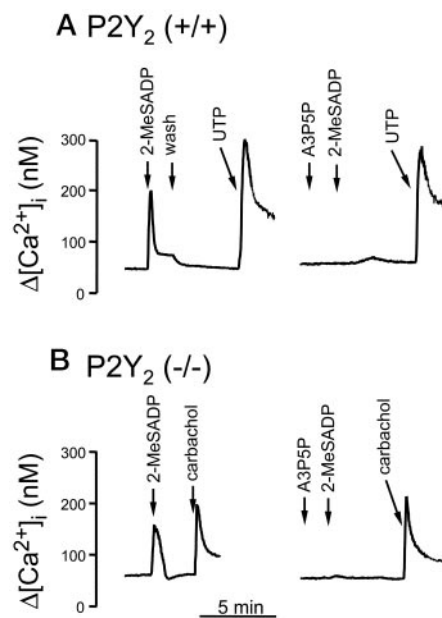
epithelial cells. These results strongly suggest that P2X receptors do not mediate the residual ATP-induced responses.

Cross-desensitization experiments with P2Y₂-R(−/−) tracheal cells indicate that the Ca²⁺$_i$ response to ATP was eliminated when the cells were pretreated with ADP or 2-MeSATP but not with UTP (Fig. 7*A*). Similarly, pretreatment with ATP or 2-MeSADP entirely abolished the 2-MeSATP-induced response in these cells (Fig. 7*B*). Studies with P2Y₂-R(−/−) nasal cells produced comparable results; pretreatment with ADP, 2-MeSATP, or 2-MeSADP abolished the ATP-induced Ca²⁺$_i$ signal (Fig. 7*C*). Similarly, the Ca²⁺$_i$ response to 2-MeSATP was eliminated when cells were pretreated with ATP, ADP, or 2-MeSADP (Fig. 7*D*). These results suggest one common receptor for ATP, ADP, 2-MeSATP, and 2-MeSADP in the P2Y₂-R(−/−) tracheal and nasal epithelial cells. This pattern of agonists resembles the nucleotide-agonist profile of P2Y₁ receptor described in many species, including the murine P2Y₁ receptor (37).

To directly investigate the involvement of the P2Y₁ receptor in P2Y₂-R(−/−) airway epithelial Ca²⁺$_i$ signaling, the effect of A3P5P, a P2Y₁ receptor-selective antagonist (38), on the 2-MeSADP-induced Ca²⁺$_i$ responses was studied (Fig. 8). 2-MeSADP (1 μM) induced substantial Ca²⁺$_i$ responses in both wild type (Fig. 8*A*) and P2Y₂-R(−/−) tracheal cells (Fig. 8*B*). The mean values for 2-MeSADP-induced changes in Ca²⁺$_i$ were 74.9 ± 23.2 nM (n = 7) and 50.4 ± 17.4 nM (n = 7) for wild type and P2Y₂-R(−/−) mice, respectively. A3P5P (100 μM) alone did not stimulate Ca²⁺ responses, but it completely blocked the effect of 2-MeSADP (Fig. 8, *right traces*). The 2-MeSADP-induced changes in Ca²⁺$_i$ in the presence of A3P5P were significantly reduced compared with responses without A3P5P: 9.8 ± 5.0 nM (n = 3) and 8.9 ± 3.7 nM (n = 4) in wild type and P2Y₂-R(−/−) cells, respectively. Similar inhibitory effects of A3P5P were observed in P2Y₂-R(−/−) nasal epithelial cells; the mean changes in Ca²⁺$_i$ in response to 1 μM 2-MeSADP were 91.2 ± 23.5 nM (n = 3) and 6.5 ± 2.5 nM (n = 3) in the absence

FIG. 8. **Effect of A3P5P on the 2-MeSADP-induced Ca²⁺ response in mouse tracheal epithelial cells.** Wild type (*A*) and P2Y₂(−/−) (*B*) mouse tracheal cells were exposed to 1 μM 2-MeSADP in the presence and absence of 100 μM A3P5P. Responsiveness of the cells was tested by adding 100 μM UTP in wild type or 1 mM carbachol in P2Y₂-R(−/−) cells at the completion of each experiment. The traces are representative of seven independent experiments.

and presence of A3P5P, respectively. In contrast, A3P5P did not affect the UTP-stimulated Ca²⁺$_i$ response in wild type cells (Fig. 8*A*), consistent with the lack of effect of A3P5P on the P2Y₂ receptor. Moreover, the carbachol-induced response in

the P2Y$_2$-R($-/-$) cells also were not affected by A3P5P (Fig. 8B), further excluding nonspecific effects of A3P5P on Ca^{2+}$_i$ signaling. Taken together, these data suggest that the residual P2 receptor in the P2Y$_2$-R($-/-$) murine airway epithelia is the P2Y$_1$ receptor.

DISCUSSION

The murine *P2Y$_2$-R* gene was disrupted by homologous recombination in embryonic stem lines and mice homozygous for the disrupted *P2Y$_2$-R* gene generated from these lines. These P2Y$_2$-R-deficient mice provide a unique tool for characterization of extracellular nucleotide regulation of cell signaling.

We investigated three different cell types isolated from lungs of wild type and P2Y$_2$ receptor (-/-) mice: lung fibroblasts and tracheal and nasal epithelial cells. Because of the absence of specific and potent antagonists, binding assays have not been useful in studies characterizing tissue-specific expression of nucleotide receptors (39). Therefore, we have measured nucleotide-induced Ca^{2+} responses and, when possible, inositol lipid hydrolysis to characterize nucleotide receptor function in cells from these wild type and P2Y$_2$ receptor-deficient mice.

A good correlation between Ca^{2+}$_i$ responses and inositol phosphate formation was observed in lung fibroblasts (Fig. 2, *A* and *B*). The dose-effect relationships for nucleotide agonists and Ca^{2+}$_i$ and inositol phosphate measurements were identical. The rank orders of agonist potencies (UTP $\geq$ ATP $\gg$ ADP > UDP) were similar in both assays and were consistent with the pharmacological profile of the P2Y$_2$ receptor. However, the recently cloned rat P2Y$_4$ receptor displays a similar pattern of triphosphate nucleotide responses (40, 41), raising the possibility that its mouse homologue may do so as well.

A definitive description of which nucleotide receptor subtype(s) accounted for the effect of UTP and ATP in the murine lung fibroblast resulted from the experiments with cells isolated from P2Y$_2$-R($-/-$) mice (Fig. 2, *C* and *D*). These data, demonstrating that disruption of the gene encoding the P2Y$_2$ receptor completely abolished nucleotide-induced inositol phosphate and Ca^{2+} responses, establish that the P2Y$_2$ is the only P2 receptor functionally expressed in mouse lung fibroblasts. Further studies will be required to extend this characterization to nonlung fibroblasts and the potential influence of continuous culture to assess the relevance of this conclusion to those in previous reports.

Because of smaller numbers and limited growth capacity of the epithelial cells, we focused on nucleotide-induced Ca^{2+}$_i$ responses rather than on inositol lipid hydrolysis in this cell type. Tracheal epithelial cells from wild type mice exhibited a rank order of nucleotide-induced responses (Fig. 3A) similar to that reported with the cloned human and mouse P2Y$_2$ receptor (7, 10). Desensitization studies carried out with wild type tracheal cells provided further support for the hypothesis that a common UTP/ATP receptor is expressed in these cells (Fig. 4).

Direct, unambiguous evidence for P2Y$_2$ expression in the murine trachea was provided by studies with P2Y$_2$-R($-/-$) tracheal cells. The complete abolition of UTP-induced Ca^{2+}$_i$ responses clearly demonstrated that P2Y$_2$ receptor accounted for the effect of UTP in wild type tracheal cells, and no other UTP-activated receptor (*i.e.* P2Y$_4$ receptor) was present (Fig. 5A). The absence of effect of UDP in the P2Y$_2$-R($-/-$) cells also ruled out involvement of P2Y$_6$ receptors. The major (75%) reduction in ATP-stimulated Ca^{2+} response clearly demonstrated that P2Y$_2$ receptor was the predominant but not unique receptor for ATP in this cell type. The reduction in the magnitude of the response to 2-MeSATP suggests that this nonselective P2Y$_1$/P2X receptor agonist also stimulates the P2Y$_2$ receptor at high concentrations. This observation is consistent with the effect of 2-MeSATP reported with the cloned P2Y$_2$ receptor

(10).

Mouse nasal epithelial cells exhibited a profile of nucleotide-stimulated responses similar to that observed in tracheal cells (Figs. 3 and 4). The ATP/UTP responses were generally larger in wild type tracheal cells than in nasal cells, whereas the magnitude of residual ATP-stimulated responses in P2Y$_2$-R($-/-$) cells was similar in cells from each region (Fig. 5). This observation suggests a higher level of expression of the P2Y$_2$ receptor in tracheal cells than nasal cells.

A second objective of our study was to identify additional nucleotide receptor(s) that might be expressed in mouse airway epithelial cells. The absence of specific agonists and antagonists for most of the P2 receptors makes it difficult to classify multiple receptors in a complex system. However, the P2Y$_2$-R($-/-$) mouse model facilitated these studies.

Our results provide direct evidence for the functional expression of P2 receptor(s) other than P2Y$_2$ that are activated by adenine nucleotide agonists in murine tracheal and nasal epithelial cells. The involvement of P1 adenosine receptors in the ATP-induced Ca^{2+} response was ruled out on the bases that adenosine did not stimulate elevation in intracellular Ca^{2+} levels and that adenosine deaminase pretreatment did not affect the response to ATP in P2Y$_2$-R($-/-$) cells. α,β-meATP, originally thought to be specific for all P2X receptors (4), is now known to be active only at P2X$_1$ and P2X$_3$ receptors (36, 42, 43). In mouse airway epithelial cells, α,β-meATP was inactive in terms of Ca^{2+} signaling (see "Results") as well as Cl$^-$ secretion (33). We cannot entirely rule out the involvement of P2X receptors solely on the basis of the absence of α,β-meATP-induced responses. However, the experiments carried out in Ca^{2+}-free buffer (Fig. 6) clearly demonstrated that ATP-stimulated Ca^{2+} responses primarily reflected release from internal stores and not direct opening of plasma membrane P2X (Ca^{2+}) channels. These findings strongly suggest that the P2X receptors are not functionally expressed in these cells.

The residual effects of ATP, ADP, 2-MeSATP, and 2-MeSADP found in P2Y$_2$-R($-/-$) cells (Fig. 5), coupled with the desensitization studies (Fig. 7), are more consistent with the expression of P2Y$_1$ receptor (44$-$46) than a P2Y$_{11}$ receptor, because the latter is not activated by the diphosphate analogues (13). The substantial Ca^{2+}$_i$ responses elicited by ADP and 2-MeSADP in the P2Y$_2$-R($-/-$) cells (Fig. 5), coupled with the antagonistic effect of A3P5P on the Ca^{2+}$_i$ response to 2-MeSADP-induced responses (Fig. 8), strongly supports the hypothesis that the residual nucleotide receptor in the P2Y$_2$-R($-/-$) airway cells is in fact the P2Y$_1$ receptor.

The results of Ca^{2+}$_i$ measurements with tracheal epithelial cells can be compared with the data obtained from the tracheal Cl$^-$ secretory studies in the accompanying paper (33). Our studies were carried out on isolated tracheal cells grown on glass coverslips, providing access of added agonists to apical and basolateral membrane surfaces. In contrast, the Cl$^-$ measurements were performed with freshly excised tracheas with additions only to the apical surface. Despite these differences, the pharmacological profiles determined by theses two methods were generally similar in tracheal specimens and revealed that the P2Y$_2$ receptor was the dominant receptor mediating both Ca^{2+}$_i$ and Cl$^-$ secretory responses in wild type mice. A slight difference was that in P2Y$_2$-R($-/-$) tracheal epithelia UDP had a minor but potent effect on Cl$^-$ secretion (33), whereas negligible UDP responses were observed in the Ca^{2+}$_i$ studies (Fig. 5). The simplest explanation for this discrepancy is that the UDP-activated receptor, probably P2Y$_6$, was down-regulated during culture on glass coverslips.

In summary, murine lung fibroblasts as well as tracheal and nasal epithelial cells from wild type mice exhibit P2Y$_2$-like

pharmacologic responses to extracellular nucleotide additions. Comparative studies of cells from P2Y$_2$-R(+/+) and P2Y$_2$-R(−/−) mice provided direct evidence for P2Y$_2$ receptor function in all three cell types. The P2Y$_2$ receptor appears likely to be the only P2 receptor in mouse lung fibroblasts and is the predominant P2 receptor in airway epithelial cells. In addition, the P2Y$_2$-R(−/−) mouse model made it possible to functionally characterize and identify another P2 receptor in airway epithelia, which was masked by the activity of the dominant P2Y$_2$ receptor. The residual nucleotide receptor in mouse tracheal and nasal epithelial cells is most likely the P2Y$_1$ receptor. Although conclusions regarding humans cannot be directly drawn from studies performed in mice, the P2Y$_2$-R(−/−) mouse model system provides a unique tool for tissue-specific nucleotide receptor function.

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