Nucleotide-regulated Calcium Signaling in Lung Fibroblasts and Epithelial Cells from Normal and P2Y₂ Receptor (−/−) Mice*

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László Homolya‡‡, William C. Watt‡, Eduardo R. Lazarowski, Beverly H. Koller, and Richard C. Boucher‡¶

From the ¶Cystic Fibrosis/Pulmonary Research and Treatment Center, University of North Carolina, Chapel Hill, North Carolina 27599 and the §Membrane Research Group of Hungarian Academy of Sciences in the National Institute of Haematology and Immunology, Budapest, H-1113, Hungary

To test for the role of the P2Y₂ receptor (P2Y₂-R) in the regulation of nucleotide-promoted Ca²⁺ signaling in the lung, we generated P2Y₂-R-deficient (P2Y₂-R(−/−)) mice and measured intracellular Ca²⁺ responses (ΔCa²⁺) to nucleotides in cultured lung fibroblasts and nasal and tracheal epithelial cells from wild type and P2Y₂-R(−/−) mice. In the wild type fibroblasts, the rank order of potencies for nucleotide-induced ΔCa²⁺ was as follows: UTP ≥ ATP > ADP > UDP. The responses induced by these agonists were completely absent in the P2Y₂-R(−/−) fibroblasts. Inositol phosphate responses paralleled those of ΔCa²⁺, in both groups. ATP and UTP also induced Ca²⁺ responses in wild type airway epithelial cells. In the P2Y₂-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 Ca²⁺, indicating that neither P1 nor P2X receptors mediated this residual ATP response. In contrast, 2-methylthio-ADP promoted a substantial Ca²⁺ response in P2Y₂-R(−/−) cells, which was inhibited by the P2Y₁ receptor antagonist adenosine 3′-5′-diphosphate. These studies demonstrate that P2Y₂-R is the dominant purinoceptor in airway epithelial cells, which also express a P2Y₁ receptor, and that the P2Y₂-R is the sole purinergic receptor subtype mediating nucleotide-induced inositol lipid hydrolysis and Ca²⁺ 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 adenosine 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 P2Y₁ receptor (P2Y₁-R). The cloning of the murine P2Y₁-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, Ca²⁺ efflux, and LDH and nucleotide release (15–19), were proposed to be mediated by A₁, A₂, P2X, P2Z (in current terminology P2X₁), and P2Y₁ receptors (15–23). In one study with human skin fibroblasts, actions of ATP on Ca²⁺ 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 Ca²⁺ 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 P2Y₁-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 P2Y₂ allele. We used these mice to examine the relative role of P2Y₂-R in the nucleotide-promoted Ca²⁺ signaling in mouse
lung fibroblasts and airway epithelial cells. The role of P2Y<sub>2</sub>-R was tested by comparison of nucleotide-stimulated Ca<sup>2+</sup> responses in cells from P2Y<sub>2</sub>-R (−/−) mice with those from wild type animals. In the accompanying paper (33), the role of P2Y<sub>2</sub>-R in mediating Cl<sup>−</sup> secretory responses in freshly excised tracheal, gallbladder, and jejunal tissues is described.

**MATERIALS AND METHODS**

*Generation of P2Y<sub>2</sub>-R-deficient Mice—* A targeting vector was designed such that DNA corresponding to base pairs 552–1149 of the published P2Y<sub>2</sub>-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 XhoI site in the 5’ region of the gene to a SmaI site located at base pair 552 of the published cDNA and a fragment extending 6500 bases 3’ from the Eco1 site at base pair 1149 of the coding sequence. The targeting vector was electroporated into E142tg21R cells, and resulting neomycin- and gancyclovir-resistant colonies were isolated. DNA from surviving colonies was isolated, digested with BamHI, and analyzed by Southern blot analysis using a probe located immediately upstream of the P2Y<sub>2</sub>-R genomic fragment not included in the targeting vector. Chimeric mice were generated with P2Y<sub>2</sub>-R-targeted E142tg21R 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<sub>2</sub>-R(+/+) and P2Y<sub>2</sub>-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 [α<sup>32</sup>P]-labeled P2Y<sub>2</sub>-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<sub>2</sub>-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<sub>2</sub>(−/−)) 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<sub>2</sub>-R(−/−) mice were euthanized with 100% CO<sub>2</sub>. Lung fibroblasts were isolated by mincing freshly excised lung parenchyma into ~1-mm<sup>3</sup> pieces and establishing explant cultures on plastic tissue culture plates in Dulbecco’s modified Eagle’s medium supplemented with 4.5 g/liter glucose. The cells were then preincubated with 10 mM Mg<sub>2+</sub>, 1.3 M Fura-2/AM for 30 min at 37 °C. After the loading period, the cells were washed twice with Hank’s balanced salt solution, 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, Nutley, NJ) at room temperature. A Zeiss Axiosvert 35 microscope and a Nikon UV-F × 100 (1.3) glycerol immersion objective were used. After each experiment, the cells were lysed with 40 µl digitonin, and the background fluorescence was determined by quenching technique using 4 mM MnCl<sub>2</sub>.

**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-[<sup>3</sup>H]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 incubation was terminated by the addition of 5% ice-cold trichloroacetic acid. The accumulated [<sup>3</sup>H]inositol phosphates were separated on Dowex AG-1X8 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 digitoxin were obtained from RBI (Natick, MA). Fura-2/AM, Fura-2-pentapotassium salt, and Ca<sup>2+</sup> calibration buffers were purchased from Molecular Probes, Inc. (Eugene, OR). myo-[<sup>3</sup>H]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 M 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<sup>2+</sup> 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<sub>max</sub>, R<sub>min</sub>, and K<sub>B</sub> values were determined by using 1 µM Fura-2 free acid and a series of Ca<sup>2+</sup> buffers. Differences between the peak and basal Ca<sup>2+</sup> concentration were plotted. The data are presented as mean ± S.E. For comparisons, the mean values were analyzed.
Purinergic Characterization of Mouse Pulmonary Cells

RESULTS

Generation of P2Y2-R-deficient Mice—Mice deficient in P2Y2-R were generated by targeted mutagenesis of the P2Y2-R gene in mouse embryonic stem cells (Fig. 1A). RNA isolated from kidneys of a P2Y2-R(+/+) and P2Y2-R(-/-) mouse confirmed the complete loss of P2Y2-R in the P2Y2-R(-/-) mouse (Fig. 1B). Mice homozygous for the mutant P2Y2-R allele were obtained at the expected frequency, were fertile, and could not be distinguished from wild type littermates. No differences were seen in 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 P2Y2-R(-/-) mice. Changes in the intracellular Ca^{2+} concentration ([Ca^{2+}]_{i}) were monitored by using Fura-2 fluorescent indicator, and nucleotide-induced [3H]inositol phosphate formation was measured in myo-[3H]inositol-labeled cells (Fig. 2). In wild type fibroblasts, ATP and UTP promoted dose-dependent Ca^{2+} responses (Fig. 2A) and inositol phosphate responses (Fig. 2B). ADP induced only a small Ca^{2+} response at high concentrations. UDP had no substantial effect.

Both Ca^{2+} and inositol phosphate responses to nucleotides were abolished in P2Y2-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 P2Y2-R receptor is the only nucleotide receptor functionally expressed in murine lung fibroblasts.

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+} 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+} responses at both 1 and 100 μM concentrations in tracheal cells from wild type mice (Fig. 3A). ADP, 2-MeSADP, and 2-MeSADP were effective only at the 100 μM concentration, whereas ATP pretreatment completely abolished the UTP-induced Ca^{2+} signal, whereas the 2-MeSADP and 2-MeSADP responses were relatively larger at the 1 μM concentration.

To investigate possible cross-desensitization between agonists in nucleotide-promoted Ca^{2+} 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+} 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+} response to ATP (Fig. 4A), whereas ATP pretreatment completely abolished the UTP-induced Ca^{2+} response (Fig. 4B). Pretreatment with ADP, and 2-MeSADP had no significant effect on ATP or UTP-induced Ca^{2+} responses (Fig. 4, A and B). The Ca^{2+} signal elicited by 2-MeSADP was entirely abolished by ATP pretreatment (Fig. 4C). UTP or 2-MeSADP pretreatment also reduced, although only partially,
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\(^{2+}\) response to 2-MeSATP was eliminated by ATP or 2-MeSATP pretreatment (Fig. 4F). 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\(_x\)-R(-/-) Mice—**A potential candidate for the common ATP/UTP receptor in airway epithelial cells is the P2Y\(_x\) receptor. To test the involvement of P2Y\(_x\) receptor in the murine airway epithelium, Ca\(^{2+}\) studies were performed on tracheal and nasal cells isolated from P2Y\(_x\)-R(-/-) mice. The UTP-induced Ca\(^{2+}\) responses were abolished in both tracheal (Fig. 5A) and nasal cells (Fig. 5B). The magnitude of ATP-stimulated Ca\(^{2+}\) responses was substantially reduced in P2Y\(_x\)-R(-/-) tracheal and nasal epithelial cells, but residual Ca\(^{2+}\) responses were measurable. The reductions in the responses to 100 \(\mu M\) ATP in wild type and P2Y\(_x\)-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\(^{2+}\) responses in both tracheal and nasal cells from P2Y\(_x\)-R(-/-) mice, whereas UDP had no substantial effect in either. A slight reduction in responses induced by 100 \(\mu M\) ADP and 2-MeSATP was observed in the P2Y\(_x\)-R(-/-) tracheal cells, but Ca\(^{2+}\) responses induced by 100 \(\mu M\) 2-MeSADP in both P2Y\(_x\)-R(-/-) tracheal and nasal cells were not significantly different from that found in wild type cells.

These results clearly demonstrate that the P2Y\(_x\) 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\(_x\)-R(-/-) Airway Epithelial Cells—**Next, we initiated a series of experiments to identify the nucleotide receptor type(s) that accounted for the residual Ca\(^{2+}\) responses induced by adenine nucleotides. To test for the involvement of adenosine receptors in ATP-promoted responses, P2Y\(_x\)-R(-/-) cells were exposed to 100 \(\mu M\) ATP in the presence or absence of 1 unit/ml adenosine deaminase. In tracheal cells, ATP stimulated a 65.4 \(\pm\) 17 nm (\(n = 3\)) change in Ca\(^{2+}\), whereas ATP-induced Ca\(^{2+}\) responses were 103.8 \(\pm\) 51 nm (\(n = 6\)) in the presence and absence of adenosine deaminase, respectively. In nasal cells, the ATP-induced Ca\(^{2+}\) responses were 108.9 \(\pm\) 29 nm (\(n = 11\)) in the presence and absence of adenosine deaminase, respectively. In addition, no Ca\(^{2+}\) response was elicited by 100 \(\mu M\) adenosine in either tracheal or nasal epithelial cells (1.5 \(\pm\) 1.0 and 4.2 \(\pm\) 0.2 nm, respectively (\(n = 3\)).

Next, we tested for the possible involvement of P2X receptors (36). To investigate this issue, 100 \(\mu M\) \(\alpha,\beta\)-meATP was applied to the P2Y\(_x\)-R(-/-) tracheal and nasal cells. No Ca\(^{2+}\) response was elicited by this compound in either cell type (\(\Delta\)Ca\(^{2+}\)) in nose, 5.2 \(\pm\) 3.9 nm, \(n = 3\); \(\Delta\)Ca\(^{2+}\) in trachea, 4.8 \(\pm\) 1.0 nm, \(n = 5\)). Further, ATP-induced Ca\(^{2+}\) 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. 6A) or P2Y\(_x\)-R(-/-) (Fig. 6B)
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+ response to ATP was eliminated when the cells were pretreated with ADP or 2-MeSATP but not with UTP (Fig. 7A). Similarly, pretreatment with ATP or 2-MeSADP entirely abolished the 2-MeSATP-induced response in these cells (Fig. 7B). Studies with P2Y2-R(−/−) nasal cells produced comparable results; pretreatment with ADP, 2-MeSATP, or 2-MeSADP abolished the ATP-induced Ca2+ signal (Fig. 7C). Similarly, the Ca2+ response to 2-MeSATP was eliminated when cells were pretreated with ATP, ADP, or 2-MeSADP (Fig. 7D). 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+ signaling, the effect of A3P5P, a P2Y1 receptor-selective antagonist (38), on the 2-MeSADP-induced Ca2+ responses was studied (Fig. 8). 2-MeSADP (1 μM) induced substantial Ca2+ responses in wild type (Fig. 8A) and P2Y2-R(−/−) tracheal cells (Fig. 8B). The mean values for 2-MeSADP-induced changes in Ca2+, 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+ 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+, 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

and presence of A3P5P, respectively. In contrast, A3P5P did not affect the UTP-stimulated Ca2+ response in wild type cells (Fig. 8A), consistent with the lack of effect of A3P5P on the P2Y2 receptor. Moreover, the carbachol-induced response in
the P2Y<sub>2</sub>-R<sup>−/−</sup> cells also were not affected by A3P5P (Fig. 8B), further excluding nonspecific effects of A3P5P on Ca<sup>2+</sup> signaling. Taken together, these data suggest that the residual P2 receptor in the P2Y<sub>2</sub>-R<sup>−/−</sup> murine airway epithelia is the P2Y<sub>1</sub> receptor.

**DISCUSSION**

The murine P2Y<sub>2</sub>-R gene was disrupted by homologous recombination in embryonic stem lines and mice homozygous for the disrupted P2Y<sub>2</sub>-R gene generated from these lines. These P2Y<sub>2</sub>-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<sub>2</sub> 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<sup>2+</sup> responses and, when possible, inositol lipid hydrolysis to characterize nucleotide receptor function in cells from these wild type and P2Y<sub>2</sub> receptor-deficient mice.

A good correlation between Ca<sup>2+</sup> responses and inositol phosphate formation was observed in lung fibroblasts (Fig. 2, A and B). The dose-effect relationships for nucleotide agonists and Ca<sup>2+</sup> and inositol phosphate measurements were identical. The rank orders of agonist potencies (UTP > ATP > ADP > UDP) were similar in both assays and were consistent with the pharmacological profile of the P2Y<sub>2</sub> receptor. However, the recently cloned rat P2Y<sub>4</sub> 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 sub-type(s) accounted for the effect of UTP and ATP in the murine lung fibroblast resulted from the experiments with cells isolated from P2Y<sub>2</sub>-R<sup>−/−</sup> mice (Fig. 2, C and D). These data, demonstrating that disruption of the gene encoding the P2Y<sub>2</sub> receptor completely abolished nucleotide-induced inositol phosphate and Ca<sup>2+</sup> responses, establish that the P2Y<sub>2</sub> 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<sup>2+</sup> 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<sub>2</sub> 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<sub>2</sub> expression in the murine trachea was provided by studies with P2Y<sub>2</sub>-R<sup>−/−</sup> tracheal cells. The complete abolition of UTP-activated Ca<sup>2+</sup> responses clearly demonstrated that P2Y<sub>2</sub> receptor accounted for the effect of UTP in wild type tracheal cells, and no other UTP-activated receptor (i.e. P2Y<sub>1</sub> receptor) was present (Fig. 5A). The absence of effect of UDP in the P2Y<sub>2</sub>-R<sup>−/−</sup> cells also ruled out involvement of P2Y<sub>6</sub> receptors. The major (75%) reduction in ATP-stimulated Ca<sup>2+</sup> response clearly demonstrated that P2Y<sub>2</sub> 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<sub>2</sub>/P2X receptor agonist also stimulates the P2Y<sub>2</sub> receptor at high concentrations. This observation is consistent with the effect of 2-MeSATP reported with the cloned P2Y<sub>2</sub> 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<sub>2</sub>-R<sup>−/−</sup> cells was similar in cells from each region (Fig. 5). This observation suggests a higher level of expression of the P2Y<sub>2</sub> 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<sub>2</sub>-R<sup>−/−</sup> mouse model facilitated these studies.

Our results provide direct evidence for the functional expression of P2 receptor(s) other than P2Y<sub>2</sub> that are activated by adenosine nucleotide agonists in murine tracheal and nasal epithelial cells. The involvement of P1 adenosine receptors in the ATP-activated Ca<sup>2+</sup> response was ruled out on the bases that adenosine did not stimulate elevation in intracellular Ca<sup>2+</sup> levels and that adenosine deaminase pretreatment did not affect the response to ATP in P2Y<sub>2</sub>-R<sup>−/−</sup> cells, α,β-meATP, originally thought to be specific for all P2X receptors (4), is now known to be active only at P2X<sub>1</sub> and P2X<sub>3</sub> receptors (36, 42, 43). In mouse airway epithelial cells, α,β-meATP was inactive in terms of Ca<sup>2+</sup> signaling (see “Results”) as well as Cl<sup>−</sup> 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<sup>2+</sup>-free buffer (Fig. 6) clearly demonstrated that ATP-stimulated Ca<sup>2+</sup> responses primarily reflected release from internal stores and not direct opening of plasma membrane P2X (Ca<sup>2+</sup>) 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<sub>2</sub>-R<sup>−/−</sup> cells (Fig. 5), coupled with the desensitization studies (Fig. 7), are more consistent with the expression of P2Y<sub>1</sub> receptor (44–46) than a P2Y<sub>11</sub> receptor, because the latter is not activated by the diphasate analogues (13). The substantial Ca<sup>2+</sup> responses elicited by ADP and 2-MeSADP in the P2Y<sub>2</sub>-R<sup>−/−</sup> cells (Fig. 5), coupled with the antagonistic effect of A3P5P on the Ca<sup>2+</sup> response to 2-MeSADP-induced responses (Fig. 8), strongly supports the hypothesis that the residual nucleotide receptor in the P2Y<sub>2</sub>-R<sup>−/−</sup> airway cells is in fact the P2Y<sub>1</sub> receptor.

The results of Ca<sup>2+</sup> measurements with tracheal epithelial cells can be compared with the data obtained from the tracheal Cl<sup>−</sup> 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<sup>−</sup> measurements were performed with freshly excised tracheas with additions only to the apical surface. Despite these differences, the pharmacological profiles determined by these two methods were generally similar in tracheal specimens and revealed that the P2Y<sub>2</sub> receptor was the dominant receptor mediating both Ca<sup>2+</sup> and Cl<sup>−</sup> secretory responses in wild type mice. A slight difference was that in P2Y<sub>2</sub>-R<sup>−/−</sup> tracheal epithelia UDP had a minor but potent effect on Cl<sup>−</sup> secretion (33), whereas negligible UDP responses were observed in the Ca<sup>2+</sup>, studies (Fig. 5). The simplest explanation for this discrepancy is that the UDP-activated receptor, probably P2Y<sub>6</sub>, 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<sub>1</sub>-like
pharmacologic responses to extracellular nucleotide additions. Comparative studies of cells from P2Y2-R(+/-) and P2Y1-R(-/-) mice provided direct evidence for P2Y2 receptor function in all three cell types. The P2Y2 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 P2Y2-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 P2Y2 receptor. The residual nucleotide receptor in mouse tracheal and nasal epithelial cells is most likely the P2Y1 receptor. Although conclusions regarding humans cannot be directly drawn from studies performed in mice, the P2Y2-R(-/-) mouse model system provides a unique tool for tissue-specific nucleotide receptor function.

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