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Effect of Loss of $P2Y_2$ Receptor Gene Expression on Nucleotide Regulation of Murine Epithelial Cl^- Transport*

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Extracellular nucleotides are believed to be important regulators of ion transport in epithelial tissues as a result of their ability to activate cell surface receptors. Although numerous receptors that bind nucleotides have been identified, the complexity of this receptor family, combined with the lack of pharmacological agents specific for these receptors, has made the assignment of particular receptors and ligands to physiological responses difficult. Because ATP and UTP appear equipotent and equieffective in regulating ion transport in many epithelia, we tested the hypothesis that the $P2Y_2$ receptor ($P2Y_2$-R) subtype mediates these responses in mouse epithelia, with gene targeting techniques. Mice with the *$P2Y_2$-R* locus targeted and inactivated ($P2Y_2$-R(−/−)) were generated, airways (trachea), gallbladder, and intestines (jejunum) excised, and Cl^- secretory responses to luminal nucleotide additions measured in Ussing chambers. Comparison of $P2Y_2$-R(+/+) with $P2Y_2$-R(−/−) mice revealed that $P2Y_2$-R mediated most (>85–95%) nucleotide-stimulated Cl^- secretion in trachea, about 50% of nucleotide responses in the gallbladder, and none of the responses in the jejunum. Dose-effect relationships for nucleotides in tissues from $P2Y_2$-R(−/−) mice suggest that the $P2Y_6$-R regulates ion transport in gallbladder and to a lesser extent trachea, whereas $P2Y_4$ and/or unidentified receptor(s) regulate ion transport in jejunum. We conclude that the $P2Y_2$ receptor is the dominant P2Y purinoceptor that regulates airway epithelial ion transport, whereas other P2Y receptor subtypes are relatively more important in other nonrespiratory epithelia.

Metabotropic (G protein-coupled) P2Y receptors are expressed in epithelia and regulate epithelial ion transport and, consequently, have become potential targets for drug therapy of diseases that reflect abnormal epithelial ion transport (1). Of particular interest in this context is cystic fibrosis (CF),[1] a disease that reflects widespread defects in epithelial ion transport due to mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) (2). However, the spectrum of epithelia affected by this disease (*e.g.* airway, gallbladder, and intestine), the diversity of P2Y receptor subtypes (3), and the absence of selective reagents to define which P2Y receptor subtypes regulate ion transport have made it difficult to initiate therapeutic programs to target specific purinoceptors in these tissues.

The strongest evidence for a role for P2 receptors in the regulation of ion transport emanates from studies of airway epithelia. Recent reports indicate that extracellular triphosphate nucleotides regulate many of the airway ion transport paths, including slowing Na^+ absorption (4) and stimulating Cl^- and K^+ secretion (5, 6). Pharmacological studies in the human airway epithelial cell line CF/T43 have demonstrated that UTP and ATP are equipotent and cross-desensitize in promoting phospholipase C activity, leading to the hypothesis that the $P2Y_2$ receptor ($P2Y_2$-R) is the dominant receptor mediating these responses (7, 8). However, it is difficult to distinguish whether the actions of ATP and UTP on ion transport are mediated by a single purine/pyrimidine-sensitive receptor (*e.g.* $P2Y_2$) and/or a combination of other recently cloned P2Y receptors that are potently activated by ATP (*e.g.* $P2Y_1$ (9, 10), $P2Y_{11}$ (11), or P2X (3, 12–14)), and UTP (*e.g.* $P2Y_4$ (15, 16)).

Studies of the P2Y receptor subtypes and regulation of ion transport in the gastrointestinal system are more limited. Data from the murine gallbladder suggest that both ATP and UTP are active in regulating Cl^- and HCO_3^- secretion (17, 18), suggesting that the $P2Y_2$-R may be the major P2Y-R subtype regulating ion transport in this tissue. Early studies of freshly excised rat jejunum reported regulation of ion transport by ATP but not UTP (19). More recent studies of triphosphate and diphosphate nucleotides in cell lines derived from the human intestine (*e.g.* CaCo2 cells) suggest that ATP, UTP, and UDP regulate enterocyte Cl^- secretory rates (20).

We report here the use of a genetic approach (21) combined with traditional pharmacological methodologies to define the relative role of purinoceptors in the regulation of ion transport in epithelial tissues. Based on the reports of the actions of ATP and UTP in the epithelia described above, a first approach was to target a single receptor that could transduce responses to ATP and UTP equipotently. The $P2Y_2$-R exhibits this pharmacology, and, consequently, mice were generated with inactivated *$P2Y_2$-R* genes using the homologous recombination technique (21). Because triphosphate nucleotide effects on Cl^- and water secretion are projected as the major therapeutic actions of these agents in epithelial diseases such as CF (5, 22), characterization of these mice focused on the role of the $P2Y_2$ receptor in mediating epithelial Cl^- secretory responses. In a companion paper (23), the role of $P2Y_2$ receptor in inositol phosphate formation and intracellular Ca^{2+} mobilization in tracheas and other tissues were characterized.

EXPERIMENTAL PROCEDURES

Mice Used for Phenotypic Characterization—Adult mice (wild type and $P2Y_2$-R(−/−)) of both sexes were used in this investigation. All

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[1] The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; I_{sc}, short circuit current; A3P5P, adenosine 3′,5′-diphosphate; α,β-meATP, α,β-methylene adenosine 5′-triphosphate; 2-MeSATP, 2-methylthioadenosine 5′-triphosphate; 2-MeSADP, 2 methylthioadenosine 5′-diphosphate, EC_{50}, 50% of maximal effective concentration.

animals were bred and raised at the University of North Carolina. The mean body mass of the normal animals (32.4 ± 1.8 g, n = 20) was not significantly different from the mean body mass of the littermate P2Y$_2$-R(−/−) mice (34 ± 1.9 g, n = 21). All mice were allowed food and water *ad libitum* until euthanized.

Mice were euthanized with 100% CO_2, and the tracheas, intestine, and gallbladders were quickly excised. The trachea was dissected free of vessels and connective tissue, split longitudinally along the posterior surface, and mounted in Ussing chambers (24). The gallbladder was opened, irrigated to remove bile, and mounted as a flat sheet in an Ussing chamber. Because of the fragility of gallbladders, a cellulose mesh was placed over the aperture of the Ussing chamber (serosal side only) to prevent the tissue from falling through the aperture. Both tissues were mounted on Ussing chambers having an aperture surface area of 0.025 cm^2. The techniques for mounting the intestinal tissue (0.25-cm^2 aperture Ussing chamber) have been previously described (25). Parafilm "O" rings were placed on both sides of the tissues to reduce edge damage.

Bioelectric Measurements—Electrical measurements of tissues were made under short circuit (I_{sc}) conditions as previously reported (24, 26). Tissues were bathed bilaterally in gassed (95% O_2, 5% CO_2) Krebs bicarbonated Ringer solution having the following composition: 140 mM Na^+, 120 mM Cl^-, 5.2 mM K^+, 1.2 mM Mg^{2+}, 1.2 mM Ca^{2+}, 2.4 mM HPO_4^{2-}, 0.4 mM H_2PO^{4-}, 25 mM HCO_3^-, and 5 mM glucose. Amiloride (10^{-4} M; Sigma) was added to the luminal side of all tracheas to block Na^+ absorption, which allows Cl^- secretory responses to agonists to be studied (24). Since the gallbladder and jejunum were unresponsive to amiloride, this drug was omitted from the protocols involving these tissues. Tissues were allowed to stabilize for 30 min before the first measurements were made or drugs added. Short circuit (I_{sc}) was continuously recorded and resistance calculated by Ohm's law by measuring the I_{sc} change in response to a 1-mV constant voltage pulse (26). All additions were to the luminal bath.

Protocols—For the dose-response studies, each dose was added cumulatively to the luminal surface of the tissue. When the tissue responded to the agonist, the response was immediate. After the response had peaked and/or stabilized (usually 2–3 min), the next dose was added. The response to the added agonist was determined as a change in the I_{sc} from the basal or previous dose level. For studies of the P2Y$_1$ antagonist A3P5P in jejunum, tissues were pretreated with A3P5P (10^{-4} M) in the luminal solution prior to nucleotide additions.

Materials—ATP and UTP were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), A3P5P was from Sigma, α,β-methylene ATP (α,β-meATP), 2-methylthio-ATP (2-MeSATP), and 2-methylthio-ADP (2-MeSADP) from RBI (Natick, MA), and UDP and adenosine from Roche Molecular Biochemicals. Because UTP could potentially contaminate commercial preparations of UDP, and to prevent formation of UTP from UDP via nucleoside diphosphokinase and endogenously released ATP, hexokinase (10 units/ml) and glucose (25 mM) were present in all UDP stocks, and the purity of UDP was monitored by high pressure liquid chromatography (27).

Data Analysis/Statistics—For all tissues, data are expressed in terms of I_{sc}. This I_{sc} response is generated primarily by Cl^- secretion and to a lesser extent HCO_3^- secretion.[2] Therefore, here the ΔI_{sc} is often referred to as Cl^- secretion. Apparent EC_{50} values represent the concentration of agonist that generated either 50% of the maximal response or, if saturation was not achieved, 50% of the response to 10^{-4} M. All data are expressed as mean ± S.E. Statistical analyses were performed with nonpaired Student's t tests.

RESULTS

Tracheal Epithelium

Characterization of Wild Type (+/+) Freshly Excised Murine Tracheal Responses to Nucleotides—The cumulative dose-effect relationships for six different nucleotides which are known to be agonists at different P_2 receptors *versus* tracheal Cl^- secretion rates (ΔI_{sc}) are shown in Fig. 1*A*; ATP and UTP were nearly equipotent and were the most effective agonists for the induction of Cl^- secretion. Table I shows that in addition to ATP and UTP, only UDP elicited a saturable Cl^- secretory response within the micromolar concentration range ($<10^{-4}$ M) (27). The maximal effect of UDP was small (~20%) compared with ATP/UTP responses. The weak effect of 2-MeSADP or 2-MeSATP suggested that it was unlikely that ATP responses were primarily mediated by a P2Y$_1$ receptor. Adenosine was also relatively ineffective (~15% of maximal ATP response; ΔI_{sc} = 36 ± 11 μA·cm^2 at 10^{-4} M, n = 5). These data suggest that the P2Y$_2$ receptor may be the dominant receptor in airway epithelia of the murine trachea but that a receptor that is activated by UDP (*e.g.* P2Y$_6$) is also expressed in this tissue.

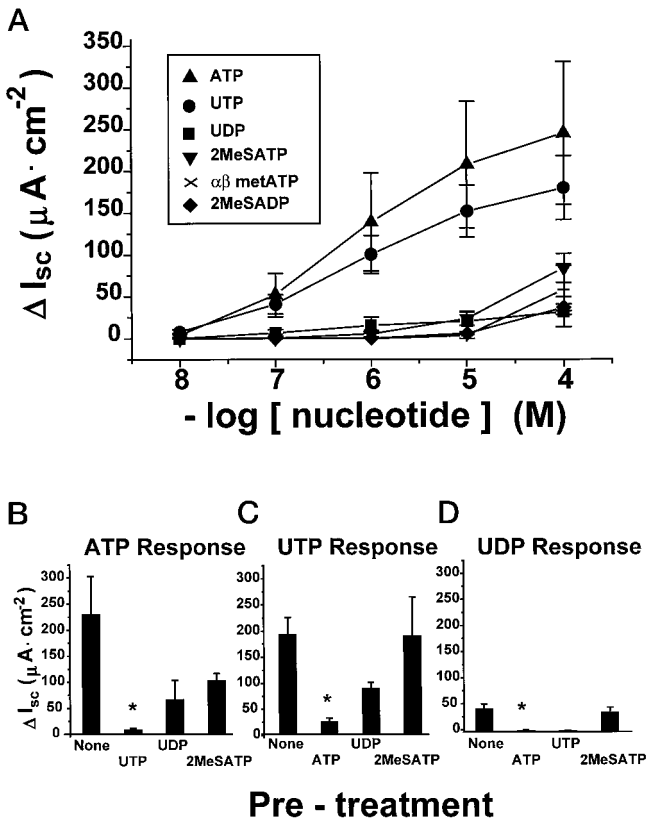

FIG. 1. **Mouse tracheas: wild type (P2Y$_2$-R(+/+)).** *A*, I_{sc} dose response to the indicated nucleotides. Data are means ± S.E., n = 6 at each dose. *B*, desensitization of tracheal ATP responses. I_{sc} responses to 10^{-4} M ATP were measured in tracheas that were pretreated with no other nucleotide (*None*, n = 7) or that were pretreated with 10^{-4} M UTP (n = 4), UDP (n = 3), or 2-MeSATP (n = 3). *C*, desensitization to UTP responses. I_{sc} responses to 10^{-4} M UTP were measured in tracheas that were pretreated with no other nucleotide (*None*, n = 7) or that were pretreated with 10^{-4} M ATP (n = 4), UDP (n = 3), or 2-MeSATP (n = 2). *D*, desensitization to UDP responses. I_{sc} responses to 10^{-4} M UDP were measured in tracheas that were pretreated with no other nucleotide (*None*, n = 6) or that were pretreated with 10^{-4} M ATP (n = 3), UTP (n = 3), or 2-MeSATP (n = 3). $p \leq 0.05$. Data in all *bar graphs* are means ± S.E.

As a second approach to identify individual P2 receptors in wild type mouse trachea, cross-desensitization studies were performed. The initial analysis of the interactions suggests that there is cross-desensitization between ATP and UTP; the ATP response was abolished by UTP pretreatment (Fig. 1*B*), and the UTP response was abolished by ATP pretreatment (Fig. 1*C*), consistent with interaction of both ligands at a single (P2Y$_2$) receptor.

However, this interpretation was complicated by the observation that both agonists produced large maximal responses (>200 μA·cm^{-2}), suggesting that the failure to observe a second response could reflect a ceiling effect and/or desensitization of other effectors in the Cl^- secretory pathway. This possibility was supported by the observations that (i) UDP responses also were abolished by ATP or UTP pretreatment (Fig. 1*D*) and (ii) the actions of the muscarinic receptor agonist carbachol were reduced after exposure of the tissue to either ATP or UTP as

[2] B. R. Grubb, unpublished observations.

TABLE I
Agonist responses in mouse tracheas

n is shown in parentheses.

Agonist	Wild-type		$P2Y_2$-R(−/−)		Decrease of maximum response
	Maximum response	Apparent EC_{50}	Maximum response	Apparent EC_{50}	
	10^{-4} M		10^{-4} M		%
ATP	229.5 ± 73 (7)	6×10^{-7}	12.7 ± 3.1 (7)	2×10^{-6}	94.8[a]
UTP	191.5 ± 37 (7)	8×10^{-7}	33 ± 9.9 (7)	2×10^{-7}	83[b]
UDP	43.6 ± 9.8 (6)	5×10^{-6}	39.4 ± 13.6 (6)	6×10^{-7}	10
α,β-meATP	58.1 ± 29 (3)	$>10^{-5}$	5.2 ± 5.2 (3)	$>10^{-5}$	91
2-MeSATP	84.5 ± 17.1 (3)	$>10^{-5}$	13.6 ± 0.8 (3)	2×10^{-6}	84
2-MeSADP	37.1 ± 3.9 (3)	$>10^{-5}$	17.2 ± 7.4 (3)	3×10^{-6}	54

[a] $p \leq 0.01$.
[b] $p \leq 0.001$.

pretreatment (Δcarbachol (10^{-4} M) naive = 62 ± 3.3 μA·cm^{-2}; Δcarbachol (10^{-4} M) post-ATP (10^{-4} M) = 19 ± 5 μA·cm^{-2}). Thus, it is not clear whether the reduced effects of UTP post-ATP indicate desensitization of a common receptor for these nucleotides (*e.g.* $P2Y_2$) or whether activation of, for example, a uridine-selective receptor (*e.g.* $P2Y_4$ or $P2Y_6$) is masked by the ceiling effect of ATP acting on an ATP-selective receptor (*e.g.* $P2Y_{11}$ or P2X receptor). Moreover, since $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors are not pharmacologically defined in the mouse, we cannot rule out the possibility that either of these receptors is activated by ATP, UTP, and UDP. The modest inhibition of the ATP response by 2-MeSATP pretreatment (Fig. 1*B*) without any effect on UTP or UDP responses (Fig. 2, *C* and *D*) raises the possibility of a low level of functional expression of a $P2Y_1$ receptor in murine trachea.

In summary, it is difficult from the above data to distinguish whether a single receptor, *e.g.* $P2Y_2$, or a mixture of receptors accounted for all of the effects observed with the six agonists tested. In an attempt to resolve these issues, a comparable characterization of the nucleotide responses of $P2Y_2$-R(−/−) mouse tracheas was performed.

Characterization of $P2Y_2$-R(−/−) Freshly Excised Tracheas—Fig. 2*A* shows the cumulative dose-effect relationships for the six nucleotides *versus* Cl^- secretion (ΔI_{sc}) for the tracheas from $P2Y_2$-R(−/−) mice plotted on the same scale as for wild type (+/+) mice. There were major (more than 85–95%) reductions in the Cl^- secretory responses to UTP and ATP at all concentrations tested. Further, there were major reductions in the magnitude of the responses to α,β-meATP, 2-MeSATP, and 2-MeSADP (Table I), indicating that these P2X and $P2Y_1$ receptor agonists (or contaminants), at high concentrations, also interact with the $P2Y_2$ receptor. No reduction in Cl^- secretory responses to UDP was observed relative to wild type, $P2Y_2$-R(+/+) mice (Table I).

To verify that the *$P2Y_2$-R* disruption did not affect the Cl^- secretory capacity of the trachea, we compared responses to carbachol. Carbachol (10^{-4} M) was equieffective in $P2Y_2$-R(+/+) (62 ± 3 μA·cm^{-2}) and $P2Y_2$-R(−/−) mice (63 ± 2 μA·cm^{-2}), suggesting that the Cl^- secretory capacity of the trachea was not decreased by targeting the *$P2Y_2$* locus ($n = 4$, both groups).

The calculated EC_{50} values, the magnitude of the maximal response, and the percentage reduction in the maximal responses for the $P2Y_2$-R(−/−) mice as compared with wild type mice (Table I) clearly indicate that the $P2Y_2$ receptor is the dominant receptor in the murine trachea that transduces the administration of triphosphate nucleotides to the tracheal epithelial surface into Cl^- secretory responses.

A variety of protocols were employed in an attempt to identify the residual receptors expressed in the $P2Y_2$-R(−/−) mouse trachea. These protocols were in general difficult because of the small magnitude of the Cl^- secretory responses mediated by residual receptors and the failure of most agonists to reach saturating concentrations within the submillimolar range.

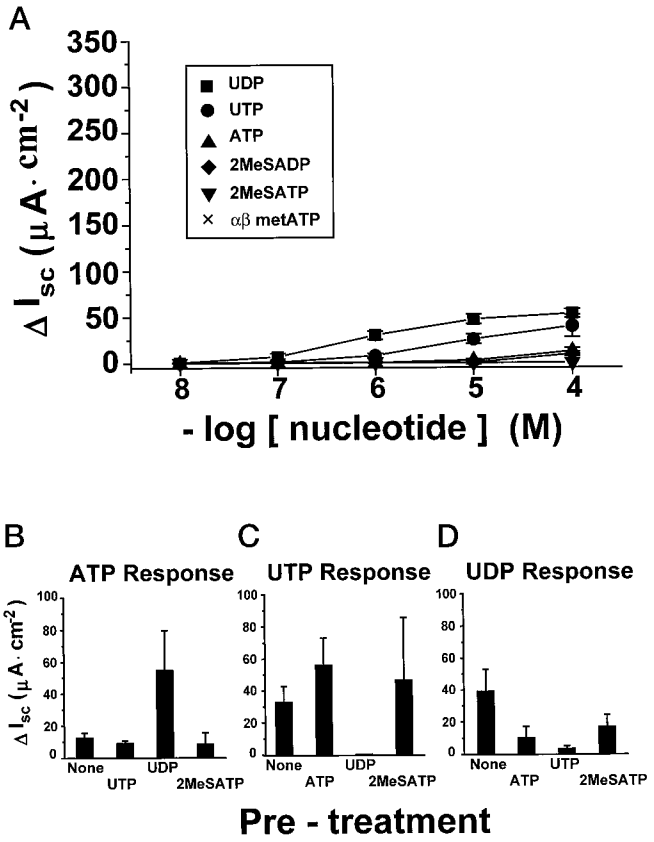

FIG. 2. **Mouse tracheas: $P2Y_2$-R(−/−).** *A*, I_{sc} dose response to the indicated nucleotides. Data are means ± S.E., $n = 6$ at each dose. *B*, desensitization to ATP responses. I_{sc} responses to 10^{-4} M ATP were measured in tracheas that were pretreated with no other nucleotide (*None*, $n = 10$) or that were pretreated with 10^{-4} M UTP ($n = 4$), UDP ($n = 3$), or 2-MeSATP ($n = 3$). *C*, desensitization to UTP responses. I_{sc} responses to 10^{-4} M UTP were measured in tracheas that were pretreated with no other nucleotide (*None*, $n = 7$) or that were pretreated with 10^{-4} M ATP ($n = 3$), UDP ($n = 3$), or 2-MeSATP ($n = 3$). *D*, desensitization to UDP responses. I_{sc} responses to 10^{-4} M UDP were measured in tracheas that were pretreated with no other nucleotide (*None*, $n = 6$) or that were pretreated with 10^{-4} M ATP ($n = 3$), UTP ($n = 3$), or 2-MeSATP ($n = 3$). Data in all *bar graphs* are means ± S.E.

UDP responses in the tracheal preparations were not affected by the $P2Y_2$ receptor disruption (Table I), suggesting that a functional $P2Y_6$ receptor is expressed in this tissue. Since $P2Y_6$ receptors are activated, although weakly, by ADP (28), a portion of the small residual ATP effect could reflect extracellular conversion of ATP to ADP (29). However, although ATP inhibits UDP-evoked responses (Fig. 2*D*), UDP does not affect subsequent responses to ATP (Fig. 2*B*). This

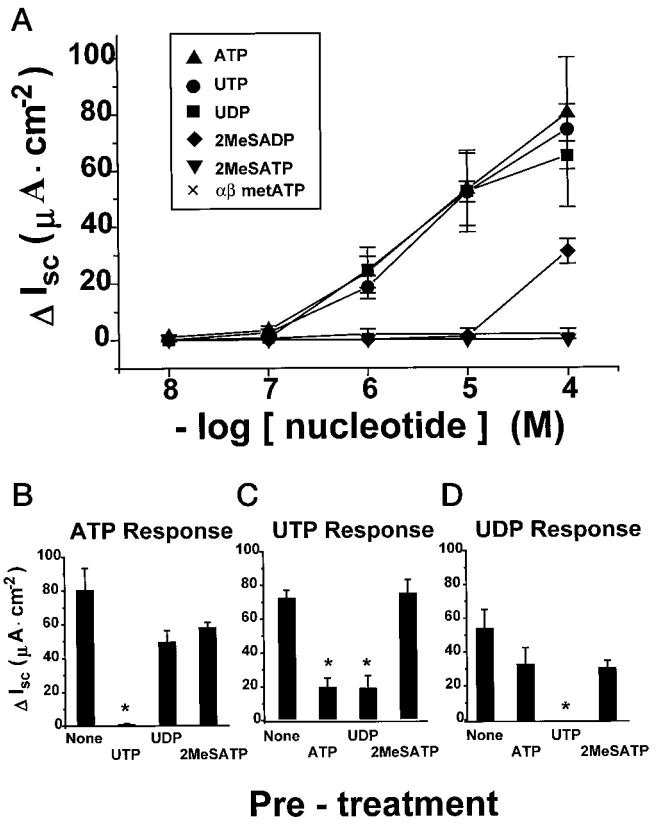

FIG. 3. **Mouse gallbladders: wild type (P2Y$_2$-R(+/+)).** *A*, I_{sc} dose response to the indicated nucleotides. Data are means ± S.E., $n = 6$ at each dose. *B*, desensitization to ATP responses. I_{sc} responses to 10^{-4} M ATP were measured in gallbladders that were pretreated with no other nucleotide (*None*, $n = 7$) or that were pretreated with 10^{-4} M UTP ($n = 4$), UDP ($n = 3$), or 2-MeSATP ($n = 3$). *C*, desensitization to UTP responses. I_{sc} responses to 10^{-4} M UTP were measured in gallbladders that were pretreated with no other nucleotide (*None*, $n = 6$) or that were pretreated with 10^{-4} M ATP ($n = 5$), UDP ($n = 3$), or 2-MeSATP ($n = 2$). *D*, desensitization to UDP responses. I_{sc} responses to 10^{-4} M UDP were measured in gallbladders that were pretreated with no other nucleotide (*None*, $n = 6$) or that were pretreated with 10^{-4} M ATP ($n = 3$), UTP ($n = 3$), or 2-MeSATP ($n = 3$). *, $p \leq 0.05$ compared with result with no nucleotide pretreatment (*None*). Data in all *bar graphs* are means ± S.E.

finding suggests that ATP could affect UDP responses by a different mechanism, *e.g.* ATP-dependent removal of UDP by ectonucleoside diphosphokinase (27).

A small residual effect of UTP was also observed in the P2Y$_2$-R −/− tracheal epithelia (Fig. 2 and Table I), which was mostly inhibited by pretreatment with UDP. This residual effect of UTP could reflect either extracellular conversion of UTP to UDP or direct activation of murine P2Y$_6$ receptors by UTP. The attenuation of UDP-promoted responses by UTP supports either of these possibilities.

Finally, the residual ATP effects were very small. The equipotency and equieffectiveness of 2-MeSADP (and 2-MeSATP) with ATP (Table I) suggest that this small residual effect may reflect a P2Y$_1$-R-like activity.

Gallbladder

Characterization of Nucleotide- stimulated Cl$^-$ Secretion in Wild Type (P2Y$_2$-R(+/+)) Freshly Excised Gallbladders—We next tested for the effect of nucleotides on the freshly excised murine gallbladder (Fig. 3). These data differ from the freshly excised trachea in one striking respect, *i.e.* UDP was virtually equieffective and equipotent with ATP and UTP in initiating a secretory response (ΔI_{sc}) in this tissue (Fig. 3*A*, Table II). 2-MeSADP induced a small response (~40% of ATP response), which was only attained at high micromolar concentrations. 2-MeSATP and α,β-meATP were entirely without effect in this tissue (Table II). Unlike the trachea, adenosine was completely ineffective ($\Delta I_{sc} = 3.9 \pm 2.8$ μA·cm^2 at 10^{-4} M, $n = 3$).

Fig. 3, *B–D*, displays cross-desensitization protocols. UTP but not UDP partially blocked the subsequent response to ATP, and ATP largely attenuated the subsequent response to UTP but not to UDP, consistent with an interaction of ATP and UTP at a P2Y$_2$ receptor. UDP attenuated the subsequent response to UTP but not to ATP (Fig. 3, *B* and *C*), and UTP blocked the subsequent response to UDP (Fig. 3*D*).

In summary, the data indicate the possible expression of a P2Y$_2$ and a P2Y$_6$ receptor in the murine gall bladder epithelium. To test this hypothesis more rigorously, we characterized the effect of nucleotides on the gallbladder epithelial cells from the P2Y$_2$-R(−/−) mouse.

Characterization of P2Y$_2$-R(−/−) Freshly Excised Gallbladder (Fig. 4)—Disruption of the *P2Y$_2$-R* gene in the gallbladder revealed that the responses to ATP were virtually abolished in the P2Y$_2$-R(−/−) gallbladders. In contrast, the effect of UTP was only partially reduced (30%) in the P2Y$_2$-R(−/−) gallbladder cells relative to the wild type cells, whereas the UDP responses were not affected by the P2Y$_2$-R disruption (Table II).

Studies of interactions with the residual ATP responses were not informative because of the very small magnitude of these residual responses (Fig. 4). With respect to UTP, the relatively large (70% of wild type) residual UTP response was blocked by UDP but not ATP (or 2-MeSATP) pretreatment (Fig. 4*C*). Similarly, the persistent UDP response was inhibited by UTP but not adenine nucleotide pre-treatment (Fig. 4*D*). These data suggest that a common receptor transduces the P2Y$_2$-R(−/−) gallbladder UDP/UTP response. The P2Y$_6$-R is a candidate for this function.

Jejunum

Effect of Nucleotides on Cl$^-$ Secretory Responses in Normal (P2Y$_2$-R(+/+)) Jejuna—The luminal dose-response curve describing the actions of ATP, UTP, ADP, and UDP on freshly excised normal mouse jejuna are shown in Fig. 5*A*. Several points deserve comment. First, unlike the tracheal and gallbladder preparations, approximately 25–30% of the normal jejunal preparations failed to respond to any nucleotide. These preparations were viable, since they had a normal response to forskolin. The nonresponding preparations were omitted from the analysis. Second, the dose-effect relationships for all the effective agonists were somewhat shifted to the right (see Table III for EC_{50} values) as compared with the activities in the gallbladder and trachea. Third, as in the other tissues, ATP and UTP were approximately equieffective and equipotent agonists at the luminal jejunum surface. Fourth, uniquely in the jejunum, ADP was also nearly as effective and potent as ATP and UTP. In contrast to the gallbladder and trachea, UDP appeared to be weak and relatively ineffective. Adenosine (10^{-4} M) was ineffective ($\Delta I_{sc} = 9 \pm 5$ μA·cm^2, $n = 3$) in jejunum. Based simply on the agonist dose-effect curves, it seems reasonable to postulate that both P2Y$_2$ (ATP/UTP)-like and perhaps P2Y$_1$-like (ADP) activities are expressed in jejuna.

Cross-desensitization experiments (Fig. 5*B*) suggested that UTP desensitized the subsequent ATP responses and that the converse was also true, consistent with the possibility that P2Y$_2$ receptors transduce ATP/UTP responses. However, the ceiling effect described earlier may be an equally likely alternative explanation for these results.

Effect of Nucleotides on Cl$^-$ Secretory Response in P2Y$_2$-R(−/−) Mouse Jejuna—In contrast to the data from the P2Y$_2$-

TABLE II
Agonist responses in mouse gallbladders

n is shown in parentheses.

Agonist	Wild-type		P2Y$_2$-R(−/−)		Decrease of maximum response
	Maximum responses	Apparent EC_{50}	Maximum responses	Apparent EC_{50}	
	10^{-4} M		10^{-4} M		%
ATP	80.3 ± 13.2 (7)	3×10^{-6}	9.9 ± 2.7 (7)[a]	1×10^{-6}	88[b]
UTP	71.3 ± 4.8 (6)	3×10^{-6}	50.5 ± 9.3 (7)	2×10^{-6}	29 NS[c]
UDP	54.6 ± 11.5 (6)	1×10^{-6}	55.1 ± 4.3 (8)	2×10^{-6}	0
α,β-meATP	1.9 ± 1.9 (3)	$>10^{-5}$	0 ± 0 (3)		100
2-MeSATP	0 ± 0 (3)		0 ± 0 (3)		
2-MeSADP	31.2 ± 4.4 (3)	$>10^{-5}$	9.7 ± 2.6 (3)[b]	$>10^{-5}$	69[d]

[a] —.
[b] $p \leq 0.001$.
[c] Not significant.
[d] $p \leq 0.01$.

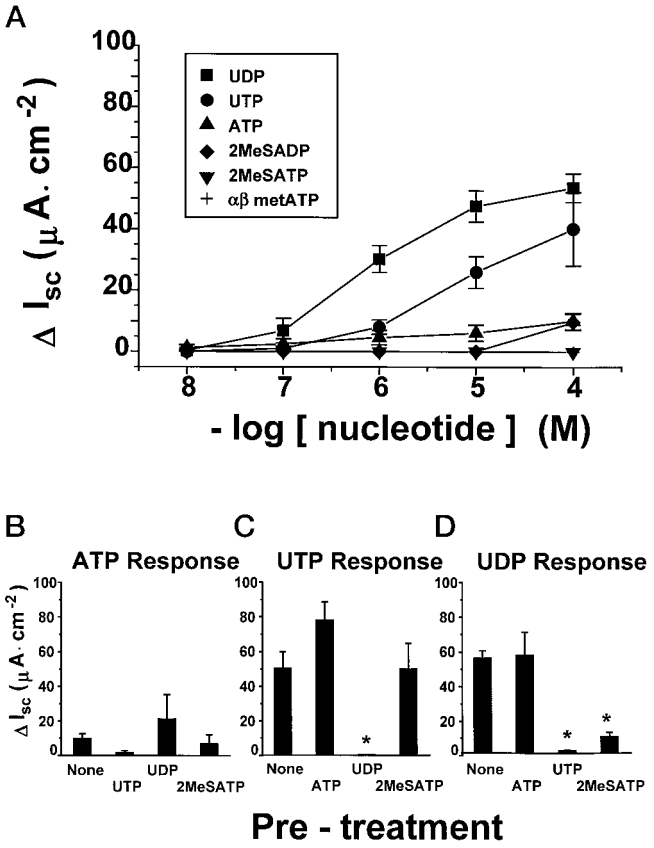

FIG. 4. **Mouse gallbladders: P2Y$_2$-R(−/−).** *A*, I_{sc} dose response to the indicated nucleotides. Data are means ± S.E., $n = 6$ at each dose. *B*, desensitization to ATP responses. I_{sc} responses to 10^{-4} M ATP were measured in gallbladders that were pretreated with no other nucleotide (*None*, $n = 7$) or that were pretreated with 10^{-4} M UTP ($n = 5$), UDP ($n = 3$), or 2-MeSATP ($n = 3$). *C*, desensitization to UTP responses. I_{sc} responses to 10^{-4} M UTP were measured in gallbladders that were pretreated with no other nucleotide (*None*, $n = 7$) or that were pretreated with 10^{-4} M ATP ($n = 6$), UDP ($n = 3$), or 2-MeSATP ($n = 2$). *D*, desensitization to UDP responses. I_{sc} responses to 10^{-4} M UDP were measured in gallbladders that were pretreated with no other nucleotide (*None*, $n = 8$) or that were pretreated with 10^{-4} M ATP ($n = 3$), UTP ($n = 3$), or 2-MeSATP ($n = 3$). *, $p \leq 0.05$ compared with pretreatment with no other nucleotide. Data in all *bar graphs* are means ± S.E.

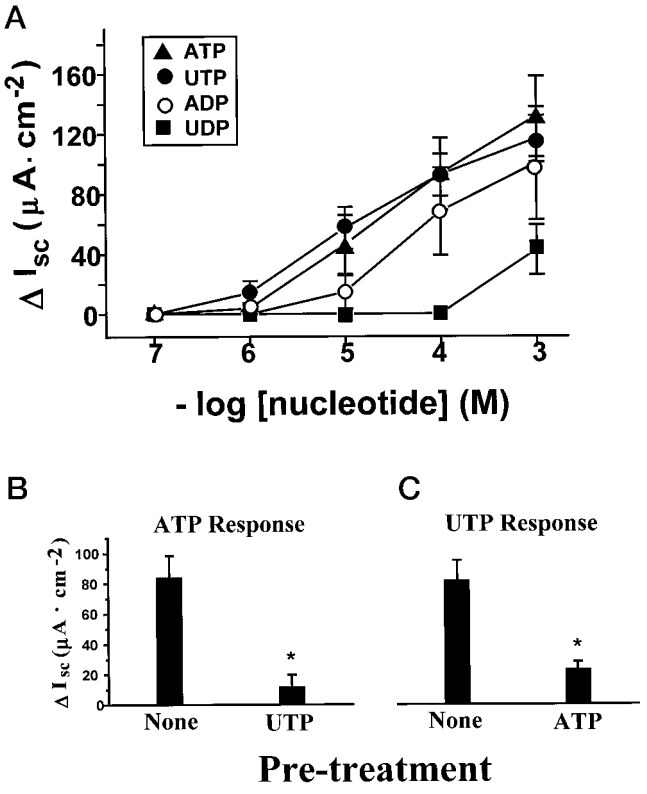

FIG. 5. **Mouse jejuna: wild type P2Y$_{2\text{-R(+/+)}}$.** *A*, I_{sc} dose response to the indicated nucleotides. $n = 6$ or more at each dose. *B*, desensitization study of response to ATP after treating tissue with 10^{-4} M UTP ($n = 5$). *None* refers to ATP response without pretreatment with another nucleotide ($n = 6$). $p \leq 0.05$. *C*, desensitization study of response to UTP after pretreating the tissue with ATP (10^{-4} M). $n = 8$. *None*, $n = 8$. *, $p \leq 0.05$.

R(−/−) trachea and gallbladder, the Cl^- secretory responses to nucleotide additions in the P2Y$_2$-R(−/−) jejuna were not different from the wild type mice (Fig. 6*A*). Like the P2Y$_2$-R(+/+) jejuna, approximately 25–30% of the P2Y$_2$-R(−/−) jejuna failed to respond to any nucleotide and were omitted from the data analyses. For the responders, the potency of ATP and UTP and the magnitude of the Cl^- secretory responses to these agonists in P2Y$_2$-R(−/−) mice were virtually identical to those of the wild type mice (see Table III).

Like the P2Y$_2$-R(+/+) jejuna, smaller responses to the second triphosphate nucleotide were observed in the P2Y$_2$-R(−/−) jejuna after the addition of a maximal dose of a first triphosphate nucleotide (Fig. 6*B*). This "desensitization" may reflect homologous desensitization of a novel P2Y$_2$-R-like receptor (see below), a ceiling effect, or heterologous desensitization between an ATP and an UTP receptor. Since pretreatment with the P2Y$_1$-R antagonist A3P5P (30) was without effect on ADP/ATP responses (Fig. 7), it is unlikely that the persistent ADP/ATP responses were mediated by P2Y$_1$-R.

DISCUSSION

Numerous studies report that both purine and pyrimidine triphosphate nucleotides regulate epithelial ion transport. Because the P2Y$_2$ receptor was the first receptor cloned that was activated by both pyrimidine and purine nucleotides, many

TABLE III
Agonist response in mouse jejunum

n is shown in parentheses.

Agonist	+/+		−/−	
	Maximum response	Apparent EC_{50}	Maximum response	Apparent EC_{50}
	10^{-3} M			
ATP	131.5 ± 27 (5)	3×10^{-5}	110.4 ± 26 (5)	2×10^{-5}
UTP	116.7 ± 15.5 (4)	10^{-5}	136 ± 40 (3)	10^{-5}
UDP	42.7 ± 16.7 (3)	3×10^{-4}	41.5 ± 6.4 (4)	3×10^{-4}
ADP	107.4 ± 35 (4)	5×10^{-5}	100 ± 37 (4)	4×10^{-5}

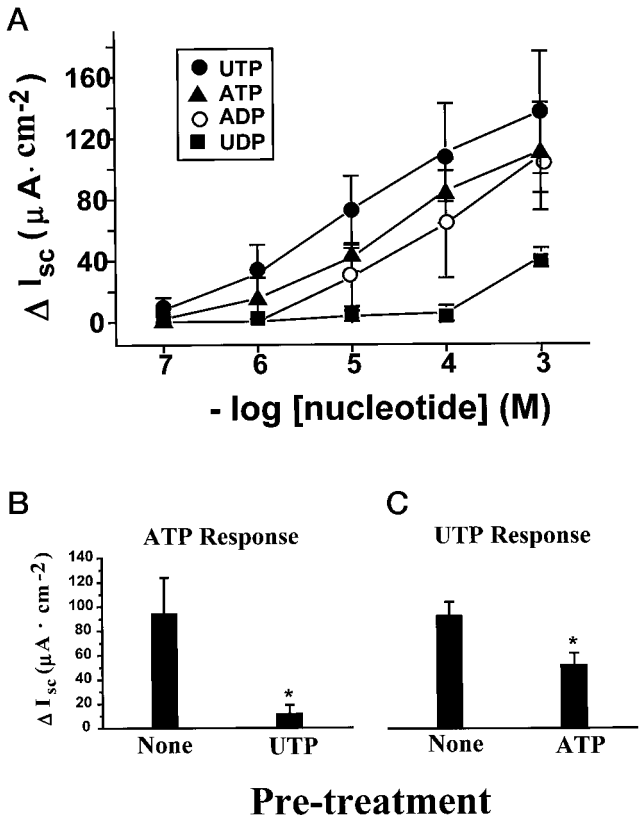

FIG. 6. **Mouse jejuna, P2Y$_2$-R(−/−).** *A*, I_{sc} dose response to the indicated nucleotides. $n = 6$ or more at each dose. *B*, desensitization study of response to ATP, after pretreating tissue with 10^{-4} M UTP ($n = 5$). *None* refers to ATP response without pretreatment with another nucleotide ($n = 5$). $p \leq 0.05$. *C*, desensitization study of response to UTP after pretreating the tissue with ATP (10^{-4} M). $n = 8$. *None*, $n = 7$. *, $p \leq 0.05$.

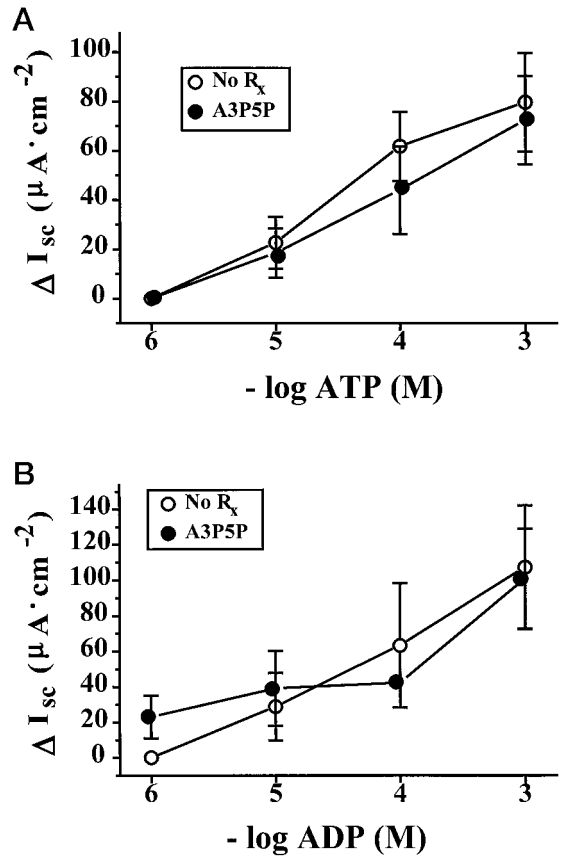

FIG. 7. **Lack of effect of P2Y$_1$-R antagonist (A3P5P) on jejunal Cl$^-$ secretory responses to adenine nucleotides.** *A*, ATP response of P2Y$_2$-R(−/−) jejuna in the presence and absence of A3P5P. *B*, ADP response of P2Y$_2$-R(−/−) jejuna in the presence and absence of A3P5P. All drugs were at a concentration of 10^{-4} M ($n = 5$ for each point).

studies have tentatively identified P2Y$_2$-R as the receptor mediating epithelial ion transport responses to triphosphate nucleotides. Based on comparisons of Cl$^-$ secretory responses in P2Y$_2$-R(+/+) and P2Y$_2$-R(−/−) mice, a surprisingly wide spectrum in the P2Y$_2$-R contribution to ATP/UTP regulated ion transport was observed in the three different epithelia studied. The contribution ranged from P2Y$_2$-R, being the dominant ATP/UTP receptor in tracheal epithelia, to the gallbladder, where P2Y$_2$-R appeared to transduce about 50% of the triphosphate nucleotide responses, to the intestine, where P2Y$_2$-R appeared to transduce virtually none of the triphosphate nucleotide actions.

These observations led to studies designed to identify the P2Y receptors in addition to P2Y$_2$-R that regulate ion transport in mouse epithelia. The following are candidate P2Y receptors that may participate in this regulation, based on reports of studies of cloned human P2 receptors: P2Y$_1$, which has a rank order potency of 2-MeSADP > ADP > 2-MeSATP > ATP (10, 31, 32); P2Y$_4$, which is activated by UTP but not by ATP (15, 16), P2Y$_6$, which has a rank order of potency of UDP ≫ ADP ≫ UTP/ATP (28, 33), and P2Y$_{11}$-R, which has a rank order potency of ATP > 2-MeSATP and is not activated by ADP, UDP, or UTP (11). A problem, however, in assigning a response to these previously characterized P2 receptors is that homologous murine P2 receptors have not been pharmacologically characterized in detail. For instance, it is not known whether UDP is an agonist at the cloned mouse P2Y$_2$ receptor (34) and what is the rank order of potencies for ATP and ADP at the cloned mouse P2Y$_1$ receptor (32). The importance of rodent data is highlighted by the recent report that the rat P2Y$_4$ receptor is activated equipotently by ATP and UTP (35, 36).

Additional concerns in a pharmacological characterization of P2 receptor activity are data indicating that agonists may not be receptor-specific. For example, α,β-meATP, which was originally thought be a specific and potent agonist for P2X receptors (37), is only a relatively weak agonist at the P2X$_1$ and P2X$_3$ receptors (38–40), while 2-MeSATP, which was originally described as a selective P2Y (P2Y$_1$) receptor agonist (37), is a

potent agonist at most P2X receptor subtypes (41). The availability of mice with the *P2Y$_2$-R* gene inactivated greatly supplemented our pharmacological approaches to identification of the roles of other P2Y receptors in nucleotide-mediated regulation of ion transport.

The concept of nucleotide-based pharmacotherapy for CF airways disease has emphasized the importance of defining the specific purinoceptor that mediates activation of Cl^- secretion in this tissue (1). The data that describe the interactions between these ligands and tracheal Cl^- secretory rates in wild type mice (Fig. 1) demonstrate the difficulty in using strictly pharmacological approaches (*e.g.* a combination of agonist dose-response curves and desensitization protocols) to assign a specific P2Y receptor subtype to an ion transport regulatory function. However, the combination of the Cl^- secretion (Figs. 1 and 2) and $Ca^{2+}{}_i$ data from a companion paper (23) comparing the wild type with P2Y$_2$-R(−/−) mice provide strong evidence for the first definitive assignment of P2Y$_2$-R to a physiologic function. Thus, although costly and time-consuming, the gene targeting technique appears to be the unique approach for unambiguously defining P2Y$_2$ receptor function in specific tissues.

There were small (~15% of P2Y$_2$-R(+/+) responses) residual ATP and UTP Cl^- secretory responses in the P2Y$_2$-R(−/−) trachea as well as a small, persistent UDP response. The detection of a relatively high potency UDP response (EC_{50} of $\sim 10^{-6}$ M, Table I) in the P2Y$_2$-R(+/+) mouse trachea and its persistence in the P2Y$_2$-R(−/−) mouse suggest that a P2Y$_6$ receptor mediated the UDP response. This observation is consistent with recent evidence of the expression of a UDP receptor in human airway epithelia (27). Data describing the equipotency of 2-MeSATP and ATP (Table I), coupled with their cross-desensitization (Figs. 1 and 2), suggest that the P2Y$_1$-R, a P2X-R, or the P2Y$_{11}$-R accounted for the very small residual ATP responses in the P2Y$_2$-R(−/−) trachea. The results of intracellular Ca^{2+} studies in the companion paper (23) favor P2Y$_1$-R as the "other" P2 receptor.

Investigations of purinoceptor regulation of gallbladder Cl^- secretion have focused on triphosphate nucleotides (17, 18, 42). In the wild type murine gallbladder, both ATP and UTP were equipotent and effective agonists, consistent with a P2Y$_2$ receptor-mediated response (Fig. 3, Table II). The effectiveness and potency of UDP was consistent with regulation of Cl^- secretion in P2Y$_2$-R(+/+) mouse gallbladder by a P2Y$_6$ receptor. An apparent major difference between the gallbladder and the trachea is that 2-MeSATP or 2-MeSADP were ineffective in gallbladder (Fig. 3), indicating that the P2Y$_1$ receptor (and P2Y$_{11}$-R) are not functionally expressed in this tissue.

The data from the P2Y$_2$-R(−/−) gallbladder indicate that virtually all of the ATP regulation of Cl^- transport is mediated via the P2Y$_2$-R. These findings were further supported by the absence of effectiveness of P2Y$_1$-R agonists and adenosine. In contrast, about 70% of the UTP response persisted. The cross-desensitization protocols with UDP and the potency order UDP > UTP suggested, but do not prove, that both the diphosphate and triphosphate pyrimidine nucleotide responses are mediated by the P2Y$_6$-R and point to the need for cloning and characterizing the mouse P2Y$_6$-R (as well as the mouse P2Y$_4$ receptor). These data suggest a role for released pyrimidines as well as purines in the regulation of gallbladder ion transport rates.

In wild type mice, the equipotency of ATP and UTP, coupled with the partial cross-desensitization, suggested that the P2Y$_2$-R transduced the Cl^- secretory responses to both purine and pyrimidine triphosphate nucleotides in jejunal epithelium (Fig. 5). We do not know why the potencies for all nucleotide agonists were shifted to the right (Fig. 5 and 6), but we speculate that this shift could reflect diffusion barriers, *e.g.* mucus, or high rates of cell surface nucleotide catabolism. These data contrast with previous studies in freshly excised rat and guinea pig intestines, which had suggested a regulatory role of ATP, but not UTP, in ion transport (19, 43). However, the data from the P2Y$_2$-R(−/−) mouse unequivocally established that these responses in mice were not transduced by the P2Y$_2$ receptor (Fig. 6; Table III). This observation requires an analysis of other P2Y receptors that transduce both purine and pyrimidine responses.

It appears unlikely that the UTP response is transduced by the P2Y$_6$ receptor, because UDP appeared to be a relatively ineffective agonist in jejunal epithelium and the UTP responses were brisk (Figs. 5 and 6). It is possible that UTP responses were transduced by a P2Y$_4$-like receptor. If the mouse P2Y$_4$-R exhibits a pharmacology similar to the rat P2Y$_4$ receptor (*i.e.* UTP = ATP), the simplest hypothesis is that mouse jejunal ion transport is functionally regulated by ATP and UTP via P2Y$_4$-R. Alternatively, if the mouse P2Y$_4$-R behaves like the human P2Y$_4$-R, *i.e.* UTP ≫ ATP, ATP and UTP might act on different receptors, which cross-desensitize each other.

The effectiveness of ADP in stimulating jejunal Cl^- secretion suggested the presence of a P2Y$_1$ receptor. However, it is unlikely that ADP (or ATP) was interacting with a luminal P2Y$_1$-R because of the failure of A3P5P to block these responses (44). Moreover, previous reports indicating a predominantly basolateral expression of P2Y$_1$-R in other epithelia (*e.g.* airways (45)) suggest that the P2Y$_1$ receptor was not involved in the Cl^- responses to luminal ADP and ATP.

There is little precedent for an ADP-stimulated response not mediated by the P2Y$_1$ receptor. The most notable action of ADP on receptors other than the P2Y$_1$ receptor occurs in platelets. ADP-promoted platelet shape change and aggregation involves three independent receptors: the P2Y$_1$ receptor, a P2X receptor, and a yet unidentified receptor that couples to inhibition of adenylyl cyclase (46). In rat C6 glioma cells, ADP also potently promotes inhibition of adenylyl cyclase by a mechanism that is independent from the P2Y$_1$ receptor (10). Besides these observations, there are no obvious additional candidates other than novel P2 receptors to mediate the jejunal response to ADP.

We conclude that the jejunal epithelium transduces Cl^- secretory responses to luminal nucleotides by a quite different set of receptors than are expressed on the tracheal or gallbladder epithelium. The effectiveness of luminal ADP in stimulating jejunal Cl^- secretion had not been reported in gut epithelia previously and was unique in the epithelia we surveyed. These observations may have important implications for potential therapies directed at this epithelium.

In summary, these studies of tissues excised from P2Y$_2$ receptor deficient mice have identified a functional role for the P2Y$_2$ nucleotide receptor subtype in the regulation of ion secretion. These studies have also demonstrated a diverse role for the P2Y$_2$-R in this activity, the contribution being dominant in airways, intermediate in the gallbladder, and absent in the jejunum. Further, by eliminating the P2Y$_2$-R from tissues, the studies have led to hypotheses that other P2Y receptor subtypes have ion transport regulatory activities in these epithelia. For example, we speculate that the P2Y$_6$-R performs a relatively large (gallbladder) and small (trachea) ion transport regulatory function in some epithelia, whereas the P2Y$_4$-like receptors are more important in others, *e.g.* jejunum. Since airways presently constitute the primary target for nucleotide therapy of CF (1) and targeting a specific P2Y receptor subtype linked to Cl^- secretion would appear to be an optimal strategy, these data support the notion of the development of potent and

selective agonists for the $P2Y_2$ receptor as potential therapies of CF lung disease and expand the spectrum of receptor targets for other epithelia.

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