Polarized expression of human P2Y receptors in epithelial cells from kidney, lung, and colon

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Wolff, Samuel C., Ai-Dong Qi, T. Kendall Harden, and Robert A. Nicholas. Polarized expression of human P2Y receptors in epithelial cells from kidney, lung, and colon. Am J Physiol Cell Physiol 288: C624–C632, 2005. First published November 3, 2004; doi:10.1152/ajpcell.00338.2004.—Eight human G protein-coupled P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14) that respond to extracellular nucleotides have been molecularly identified and characterized. P2Y receptors are widely expressed in epithelial cells and play an important role in regulating epithelial cell function. Functional studies assessing the capacity of various nucleotides to promote increases in short-circuit current (Isc) or Ca2+ mobilization have suggested that some subtypes of P2Y receptors are polarized with respect to their functional activity, although these results often have been contradictory. To investigate the polarized expression of the family of P2Y receptors, we determined the localization of the entire P2Y family after expression in Madin-Darby canine kidney (MDCK) type II cells. Confocal microscopy of polarized monolayers revealed that P2Y1, P2Y11, P2Y12, and P2Y14 receptors reside at the basolateral membrane, P2Y2, P2Y4, and P2Y6 receptors are expressed at the apical membrane, and the P2Y13 receptor is unsorted. Biotinylation studies and Isc measurements in response to the appropriate agonists were consistent with the polarized expression observed in confocal microscopy. Expression of the Gq-coupled P2Y receptors (P2Y2, P2Y4, P2Y6, and P2Y11) in lung and colonic epithelial cells (16HBE14o− and Caco-2 cells, respectively) revealed a targeting profile nearly identical to that observed in MDCK cells, suggesting that polarized targeting of these P2Y receptor subtypes is not a function of the type of epithelial cell in which they are expressed. These experiments highlight the highly polarized expression of P2Y receptors in epithelial cells.

Madin-Darby canine kidney; 16HBE14o−; Caco-2; confocal microscopy; polarized targeting

EXTRACELLULAR NUCLEOTIDES such as ATP and UTP are released from essentially all cells and interact with cell surface P2 receptors to produce a broad range of physiological responses. P2 receptors are divided into two major classes: P2X receptors, which are nonselective cation channels, and P2Y receptors, which are members of the G protein-coupled receptor (GPCR) superfamily (14, 36). Molecular cloning and characterization studies have identified eight functional human G protein-coupled P2Y receptor subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14), which fall into two subfamilies on the basis of their signaling properties and sequence identities. The P2Y1 receptor family, comprising P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 receptors, activates heterotrimeric G proteins of the Gi family, thereby activating phospholipase C and promoting inositol lipid-dependent signaling (14, 36). In addition to activating Gi, the P2Y11 receptor also activates Gq and therefore stimulates adenylyl cyclase activity (5, 35, 40). The P2Y12 receptor subfamily, comprising P2Y12, P2Y13, and P2Y14 receptors, activates Gs, thereby promoting inhibition of adenylyl cyclase activity (3, 4, 15, 46).

Although P2Y receptors regulate multiple physiological processes in a variety of cells and tissues, one of their major roles is in the regulation of ion transport and stress responses in epithelial cells (18, 26). Epithelial cells line the interstitial surfaces in the lung, kidney, and intestine and create a barrier between the external environment and the underlying cells and tissue. This paracellular barrier is created by a complex of proteins known as the tight junction, which forms an intercellular connection that creates a monolayer impermeable to water and ions. Tight junctions also serve to demarcate two distinct membrane surfaces in polarized epithelial cells: the apical surface, which lies above the tight junction and faces the lumen, and the basolateral surface, which lies below the tight junction and contacts underlying cells. The differential expression of membrane proteins, including P2Y receptors, at one of these two surfaces allows these cells to regulate a broad range of homeostatic functions, including the movement of water, ions, and nutrients between the lumen and underlying tissue (43).

G protein-coupled P2Y receptors serve an important role in autocrine and paracrine regulation of ion and nutrient transport in epithelial cells. The first indication that P2Y receptors served in this capacity was the observation that ATP and UTP, when added to the apical surface of airway cells, promoted a Ca2+-activated Cl− current (28). Multiple subsequent studies showed that all five of the subtypes in the P2Y1 receptor family are expressed in epithelial cells from various tissues (8, 17, 26, 27, 34, 38, 42, 44, 45). Moreover, many of these epithelial cells express multiple subtypes of P2Y receptors (33). Although mRNAs encoding P2Y12 and P2Y14 receptors have been observed in tissues containing epithelial cells, direct demonstration of receptor expression in epithelial cells has not yet been reported. These studies demonstrated that all five Gq-coupled subtypes of P2Y receptors (and potentially the Gi-coupled subtypes) are expressed in epithelial cells and highlight the prominent role of extracellular nucleotides in regulation of epithelial cell function.

Many of the aforementioned studies suggested that P2Y receptors are localized to distinct membrane surfaces in polarized epithelial cells. However, with the exception of the canine P2Y11 receptor, in which a receptor-green fluorescent protein

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(GFP) fusion protein was shown to be targeted to the basolateral membrane of Madin-Darby canine kidney (MDCK) cells (44), most of these studies have addressed the question of P2Y receptor polarization in an indirect manner or with potentially nonspecific antibodies that lend uncertainty to the conclusions regarding polarized targeting. To avoid these mitigating factors and to define the targeting properties of the entire family of P2Y receptors in epithelial cells, we determined the steady-state localization of P2Y receptors by visualizing hemagglutinin (HA)-tagged receptors expressed in MDCK type II [MDCK(II)] cells (ATCC, Rockville, MD) using confocal microscopy. These studies were further supplemented by quantification of receptor distribution by using biotinylation and measurement of agonist-induced changes in short-circuit current (Isc). Remarkably, our data indicate that all but one of the eight P2Y receptors are localized exclusively to either the apical or basolateral membrane surfaces of MDCK(II) cells. Moreover, a targeting profile nearly identical to that of the Gq-coupled P2Y receptor family in MDCK cells was obtained in lung 16HBE14o– and colonic Caco-2 cells, suggesting that targeting of P2Y receptors is not a function of the cell line in which they are expressed. This is the first study to define the targeting properties of the entire family of P2Y receptors in polarized epithelial cells.

MATERIALS AND METHODS

Construction of HA-tagged receptor constructs. Human P2Y receptor cDNA constructs were amplified by PCR with Phusion polymerase (Stratagene, La Jolla, CA) from HeLa cell genomic DNA. The upstream primers were complementary to codons 2–8 of each individual P2Y receptor, whereas the downstream primers were reverse complementary to the last six codons and the stop codon of each coding sequence. To aid in subcloning, the 5′ primers contained an MluI site immediately preceding the second codon of the individual receptor, whereas the 3′ primers contained an XhoI site following the stop codon. The 5′ primer for the P2Y1 receptor, which is the only P2Y receptor whose coding sequence is interrupted by an intron, comprised an MluI site, codons 2–4 from the first exon, and codons 5–11 of the second exon (5). The amplified cDNAs were digested with MluI and XhoI and ligated in-frame into a similarly digested pLXSN retroviral expression vector containing a Kozak initiation sequence, initiating methionine residue, and the HA epitope tag (YPYDVPDY).

Cell culture and expression of receptor constructs. MDCK(II) cells were subcultured in DMEM/F-12 (1:1 medium) (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS; HyClone, Gaithersburg, MD) and 1× penicillin/streptomycin in a humidified incubator at 37°C with 5% CO2-95% air. 16HBE14o– cells, from an immortalized human bronchial epithelial cell line (7), were grown on collagen-coated plates in MEM (Invitrogen) supplemented with 10% FBS, 1% sodium pyruvate, 1% nonessential amino acids, and 1× penicillin/streptomycin. Caco-2 cells (11), from an immortalized human colonic epithelial cell line, were grown in the same medium as 16HBE14o– cells, except that the FBS concentration was increased to 20%.

Recombinant retroviral particles were produced by calcium phosphate-mediated transfection of PA317 cells with pLXSN vectors containing HA-tagged human P2Y (hP2Y) receptor constructs as previously described (6) and were used to infect the various cell lines. Geneticin-resistant cells were selected after 7–10 days with 1 mg/ml G418 and maintained in medium containing 0.4 mg/ml G418.

Confocal microscopy. MDCK(II), 16HBE14o–, and Caco-2 cells stably expressing HA-tagged hP2Y receptor constructs were seeded (6 × 10⁵ cells/well) in 12-mm polyester Transwell inserts (0.4 μm; Corning Life Sciences, Acton, MA). All cells were allowed to polarize for 5–7 days with daily medium changes. Cell monolayers were washed with cold PBS+ (phosphate-buffered saline containing 2 mM Ca2+ and Mg2+), fixed and permeabilized with −20°C methanol for 4 min, and blocked with PBS containing 1% nonfat dry milk for 30 min at room temperature. Receptors were labeled with anti-HA mouse monoclonal antibody HA.11 (Covance, Berkeley, CA), and tight junctions were labeled with a rabbit polyclonal antibody to zonula occludens-1 (ZO-1; Zymed, South San Francisco, CA). Cells were washed three times with cold PBS++ and then labeled with goat anti-mouse A-488 (for P2Y receptors) and goat anti-rabbit A-594 (for ZO-1) secondary antibodies (Molecular Probes, Eugene, OR). The fixed and stained monolayers were washed several times with cold PBS++, excised from the Transwell inserts, and mounted on glass microscope slides with Slowfade mounting medium (Molecular Probes).

Confocal microscopy was performed on an Olympus Fluoview 300 laser scanning confocal imaging system (Melville, NY) configured with an IX70 fluorescence microscope fitted with a PlanApo ×60 oil objective. Multiple XY (horizontal to the monolayer) and XZ (vertical to the monolayer) scans were acquired for each monolayer.

Quantitation of cell surface HA-tagged P2Y receptors. MDCK(II) cells stably expressing HA-tagged hP2Y receptors were seeded in duplicate in 24-mm Transwell inserts and allowed to polarize as described in Confocal microscopy. Monolayers were plated on ice and kept at 4°C for the duration of the experiment. Cells were washed with cold PBS+++ three times for 5 min each and then labeled with 1 mg/ml sulfo-NHS-SS-biotin (Pierce, Rockford, IL) in cold PBS++ buffer, pH 8, for 40 min. The biotin solution was removed, and the reaction was quenched with 100 mM glycine in PBS++ for 10 min. The cells were washed and then incubated for 5 min with 0.7 ml Tris-Triton buffer (50 mM Tris·HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, and 1% Triton X-100) containing a protease inhibitor cocktail. The cells were washed 7–10 times through a 25-gauge needle and then incubated for 1.5 h with rocking. The cell lysate was centrifuged at 20,000 g for 30 min, and the supernatant was incubated with 50 μl of immobilized Neutradvin (Pierce) for 1.5 h. The resin was washed twice with Tris-Triton buffer, and biotin-labeled proteins were eluted from the Neutradvin resin by incubation with 35 μl of SDS-PAGE sample buffer containing 100 mM diethiothreitol for 10 min at 37°C. The diethiothreitol cleaves the disulfide within the biotin spacer and releases the proteins from Neutradvin under mild conditions.

Eluted proteins were separated by SDS-PAGE on a 10% gel and transferred overnight to nitrocellulose membranes. Membranes were blotted via a standard Western blotting protocol with the anti-HA monoclonal antibody conjugated to horseradish peroxidase (3F10; Roche Biochemicals, Indianapolis, IN). The blots were developed with SuperSignal West Pico chemiluminescent substrate (Pierce), and the resulting bands were imaged on a Bio-Rad Fluor-S system and quantitated with Bio-Rad QuantityOne software (Bio-Rad, Hercules, CA).

Radioligand binding assay. A binding assay for membranes was performed as previously described (41). Briefly, various concentrations of MDCK(II) membranes were incubated with an approximate Kd concentration (8 nM) of [3H]MRS2279 for 30 min at 4°C. Binding reactions were terminated by the addition of 4 ml of cold assay buffer (20 mM Tris·HCl, pH 7.5, 145 mM NaCl, and 5 mM MgCl2) and filtered through GF/A filters to retain membrane-bound [3H]MRS2279. Filters were washed once with cold assay buffer and placed in scintillation fluid for measurement of radioactivity. Specific binding of 8 nM [3H]MRS2279 to MDCK membranes was determined as total radioligand bound minus the radioligand bound in the presence of 30 μM MRS2179, a P2Y1 receptor-selective antagonist (2).

Using chamber measurement of Isc. MDCK(II) cells stably expressing HA-tagged hP2Y receptors were seeded in 12-mm polyester Snapwell inserts (Corning Life Sciences) and allowed to polarize for
5–7 days as described in Confocal microscopy. The inserts were placed in Ussing chambers and monitored for changes in $I_{sc}$ in response to cumulative concentrations of the appropriate nucleotides added to either the mucosal (apical) or serosal (basolateral) surface. The maximal response at each concentration was plotted as a cumulative increase in $I_{sc}$ vs. nucleotide concentration.

**RESULTS**

**Localization of HA-tagged P2Y receptors in MDCK(II) cells.** To determine the membrane targeting of the entire family of P2Y receptors in epithelial cells, we individually expressed HA-tagged constructs of P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14 receptors in MDCK(II) cells by retroviral infection and then determined the steady-state localization of each receptor in polarized monolayers by confocal microscopy. MDCK(II) cells have been used extensively as a model cell line to define the targeting properties of a broad range of membrane proteins (20, 30, 31). MDCK(II) cells expressing each P2Y receptor were cultured in Transwells and allowed to polarize for 5–7 days. The cells were then fixed and labeled with antibodies directed against either the HA epitope or ZO-1 (a marker of the epithelial tight junction) as described in MATERIALS AND METHODS.

Figure 1 shows $XY$ and $XZ$ cross sections of wild-type MDCK(II) cells and MDCK(II) cells expressing each of the eight P2Y receptor subtypes. Wild-type MDCK(II) cells showed staining of the tight junctions but no staining with the anti-HA antibody, demonstrating the specificity of both antibodies in MDCK(II) cells. Confocal micrographs of MDCK(II) cells expressing each P2Y receptor revealed that seven of the eight receptor subtypes were localized at steady state to either the apical or basolateral membrane surface. P2Y1, P2Y11, P2Y12, and P2Y14 receptors were expressed heavily along the lateral regions of the cell below the tight junction with a low level of expression at the basal membrane. Essentially no
visible staining for these receptors was observed in the apical membrane. In contrast, P2Y2, P2Y4, and P2Y6 receptors were expressed exclusively at the apical membrane, with little to no staining below the tight junction (Fig. 1). The only receptor that was not localized was the P2Y13 receptor. Thus the family of P2Y receptors shows a distinct pattern of polarized expression in MDCK(II) cells.

**Biotinylation of P2Y receptors in MDCK(II) cells.** To provide a more quantitative measure of receptor polarization, we utilized a biotinylation assay to determine the levels of receptor expression at either the apical or basolateral membrane. MDCK(II) cells expressing each P2Y receptor were biotinylated from either the apical or basolateral surface, and biotinylated receptors were quantified as described in MATERIALS AND METHODS. Representative blots are shown in Fig. 2, and the percentages of biotinylated receptors at each membrane surface are presented in Table 1. Consistent with the confocal images, all of the receptor subtypes except the P2Y13 receptor showed a strongly polarized steady-state localization, with ≥96% of P2Y1, P2Y11, P2Y12, and P2Y14 receptors expressed on the basolateral surface and ≥97% of the P2Y2, P2Y4, and P2Y6 receptors expressed on the apical surface. The P2Y13 receptor was expressed at similar levels at both membrane surfaces.

**Localization of the P2Y1 receptor subfamily in 16HBE14o− and Caco-2 cells.** To confirm that polarized expression of P2Y receptors in MDCK(II) cells is not a cell-specific phenomenon, we also expressed HA-tagged P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 receptors in two immortalized human epithelial cell lines, 16HBE14o− bronchial epithelial cells (1, 7, 13) and Caco-2 colonic adenocarcinoma cells. As in MDCK(II) cells, P2Y1 and P2Y11 receptors were expressed at the basolateral surface in 16HBE14o− and Caco-2 cells, whereas P2Y4 and P2Y6 receptors were expressed at the apical membrane (Fig. 3). The P2Y2 receptor was expressed at the apical membrane in both 16HBE14o− and Caco-2 cells, but in 16HBE14o− cells the receptor also was expressed at lower levels in the lateral membrane below the tight junctions (Fig. 3). These data demonstrate that the Gq-coupled P2Y receptors are targeted in an essentially identical manner in epithelial cells derived from three distinct tissues, suggesting that targeting of the Gq-coupled P2Y receptor subtypes is not dependent on the type of epithelial cell in which they are expressed.

**Functional expression of P2Y receptors in MDCK(II) cells.** Progress in molecular and physiological studies of the P2Y receptors has been hampered by the lack of reliable radioligands for their quantification. However, Waldo et al. (41) recently developed [3H]MRS2279 as a radioligand that is effective for quantification of natively and exogenously expressed P2Y1 receptors. This radioligand was utilized to quantify P2Y1 receptors in wild-type MDCK(II) cells and after stable expression of the human P2Y1 receptor. P2Y1 receptor levels increased from 8 ± 1 fmol/mg protein for the endogenous canine P2Y1 receptor of wild-type MDCK(II) cells to 185 ± 5 fmol/mg protein after selection of a population of MDCK cells stably expressing the exogenous human P2Y1 receptor.

The functional activity of the exogenous P2Y1 receptor also was examined in polarized MDCK(II) cells by measuring Isc in Ussing chambers across monolayers of wild-type cells or cells expressing the P2Y1 receptor (Fig. 4). Isc is the summation of the flow of both cations and anions through multiple channels across a monolayer of cells. Increasing concentrations of 2-methylthio-ADP (2-MeSADP) were added cumulatively to either the apical or basolateral compartments, and Isc was measured. Interpretation of these experiments was complicated by the endogenous expression in MDCK cells of the canine homologs of P2Y1, P2Y2, and P2Y11 receptors (34, 44), which give rise to increases in Isc in the absence of exogenous expression of human P2Y receptors. Thus we relied on the observation that concentration-response curves for agonists shift to the left as a function of increases in GPCR expression (21). This procedure has been utilized previously by Zámbon et al. (44) in their studies with the canine P2Y11 receptor.

Consistent with our confocal and biotinylation experiments, overexpression of the P2Y1 receptor in MDCK(II) cells pro-

### Table 1. Quantification of cell surface expression of P2Y receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Apical (fmol/mg protein)</th>
<th>Basolateral (fmol/mg protein)</th>
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<tr>
<td>P2Y1</td>
<td>1 ± 0.3</td>
<td>99 ± 0.3</td>
</tr>
<tr>
<td>P2Y2</td>
<td>97 ± 2</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>P2Y4</td>
<td>98 ± 2</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>P2Y6</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>P2Y11</td>
<td>4 ± 2</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>P2Y12</td>
<td>2 ± 2</td>
<td>98 ± 2</td>
</tr>
<tr>
<td>P2Y13</td>
<td>54 ± 1</td>
<td>46 ± 1</td>
</tr>
<tr>
<td>P2Y14</td>
<td>1 ± 0.3</td>
<td>99 ± 0.3</td>
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Cell-surface expression of P2Y receptors at the apical and basolateral membrane surfaces was quantitated using a polarized biotinylation assay. P2Y receptor-expressing Madin-Darby canine kidney type II [MDCK(II)] cells were labeled with biotin from either the apical or basolateral surface, and labeled receptors were quantitated as described in MATERIALS AND METHODS. Values are means ± SE averaged over 3 experiments.
moted a 12-fold leftward shift in the concentration-response curve for 2-MeSADP at the basolateral membrane (Fig. 4 and Table 2). Interestingly, this shift to the left in the concentration-response curve for 2-MeSADP was similar to the ~20-fold increase in P2Y1 receptor density quantified by [3H]MRS2279 binding. Moreover, both the magnitude and potency of the 2-MeSADP-promoted apical currents were considerably lower than those promoted by basolaterally applied 2-MeSADP. The increase in \( I_{sc} \) following apical application of 2-MeSADP in MDCK(II) cells expressing the human P2Y1 receptor likely represents a small amount of expression of the receptor at the apical surface, again consistent with our confocal and biotinylation studies.

Similar results were obtained with the other \( G_{q} \)-coupled receptor-expressing cells. That is, overexpression of the P2Y receptor in MDCK(II) cells resulted in a leftward shift in the concentration-response curves for the appropriate agonists (UTP for P2Y2- and P2Y4-expressing cells, UDP for P2Y6-expressing cells, and ATP for P2Y11-expressing cells) at the membrane surface to which the receptor was primarily targeted (Fig. 4 and Table 2). Unusual results were observed with the P2Y11 receptor, because the ATP response at the apical membrane of wild-type MDCK(II) cells decreased significantly after expression of the P2Y11 receptor. However, the basolateral ATP responses (where the P2Y11 receptor is exclusively expressed; Fig. 1) were entirely consistent with those obtained with the other receptors. In contrast to the five \( G_{q} \)-coupled subtypes, we did not observe changes in \( I_{sc} \) in cells expressing \( G_{i} \)-coupled P2Y12, P2Y13, or P2Y14 receptors, suggesting that \( G_{i} \)-mediated pathways are not involved in regulating epithelial \( I_{sc} \) under our experimental conditions.

**DISCUSSION**

Although much work has been carried out on membrane protein localization in polarized epithelial cells, a relative paucity of data exists documenting the polarization of GPCRs. In the current study, we examined the polarization of the entire family of P2Y receptors, many of which are known to play important roles in the function and regulation of epithelial cells. Our data demonstrate that seven of the eight members of the family of P2Y receptors are expressed in a highly polarized manner in MDCK(II) epithelial cells, and these results were verified in both biotinylation studies and \( I_{sc} \) measurements in live cells. This is the first study to visualize directly the steady-state localization of the entire P2Y receptor family with confocal microscopy.

The marked polarized distribution of seven of the eight P2Y receptor subtypes was striking. In contrast, only two of the five muscarinic receptor subtypes, M2 and M3, are targeted to distinct membrane domains in MDCK(II) epithelial cells (31). Polarization of P2Y receptors may result from the fact that all five of the \( G_{q} \)-coupled receptors (17, 25, 42, 44) [and potentially 2 of the 3 \( G_{i} \)-coupled receptors (3, 15)] are natively expressed in polarized cell types, i.e., epithelial and endothelial cells, where targeting of receptors to distinct membrane sur-
faces is critical for proper function. These data suggest that the seven polarized receptors contain targeting signals that direct the protein to either the apical or basolateral surface. Therefore, receptors not known to be expressed endogenously in epithelial cells, such as the P2Y13 receptor, might lack the proper targeting information to ensure a polarized distribution and by default have an unsorted phenotype. Our data are consistent with this idea.

Table 2. EC50 values for P2Y regulation of Isc

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Nucleotide</th>
<th>EC50 of Wild-Type Cells, μM</th>
<th>EC50 of P2Y-Expressing Cells, μM</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>AP</td>
<td>BL</td>
</tr>
<tr>
<td>P2Y1</td>
<td>2-MeSADP</td>
<td>NR</td>
<td>1.6±0.1</td>
</tr>
<tr>
<td>P2Y2</td>
<td>UTP</td>
<td>6.2±0.1</td>
<td>ND†</td>
</tr>
<tr>
<td>P2Y4</td>
<td>UTP</td>
<td>6.2±0.1</td>
<td>ND</td>
</tr>
<tr>
<td>P2Y6</td>
<td>UDP</td>
<td>ND</td>
<td>NR</td>
</tr>
<tr>
<td>P2Y11</td>
<td>ATP</td>
<td>3.3±0.1</td>
<td>ND</td>
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Wild-type and P2Y receptor-expressing MDCK(II) monolayers were challenged with cumulative doses of the appropriate nucleotides in Ussing chambers, and the resulting Isc was measured. Isc in response to increasing concentrations of the indicated nucleotide are plotted for both WT and P2Y receptor-expressing MDCK(II) cells and are representative traces of 3 independent experiments. EC50 values (μM) of the indicated nucleotides are shown in Table 2. 2MeSADP, 2-methylthio-ADP.

Fig. 4. Measurement of short-circuit current (Isc) in WT and P2Y receptor-expressing MDCK(II) cells. WT and P2Y receptor-expressing MDCK(II) monolayers were challenged with cumulative doses of the appropriate nucleotides in Ussing chambers, and the resulting Isc was measured. Isc in response to increasing concentrations of the indicated nucleotide are plotted for both WT and P2Y receptor-expressing MDCK(II) cells and are representative traces of 3 independent experiments. EC50 values (μM) of the indicated nucleotides are shown in Table 2. 2MeSADP, 2-methylthio-ADP.

Fig. 5. The P2Y receptor family and their polarized distribution in MDCK(II) epithelial cells. The family of P2Y receptors and their cognate agonists are shown with their localization in polarized MDCK(II) cells. Receptors expressed at the apical surface (P2Y2, P2Y4, and P2Y11) are shaded red, whereas those expressed at the basolateral surface (P2Y1, P2Y12, P2Y13, and P2Y14) are shaded blue, and the unsorted receptor (P2Y13) is gray. The dashed line demarcates the division between the apical and basolateral membrane domains. Receptors that are adenine nucleotide selective are expressed at the basolateral surface, whereas receptors that are activated by uridine nucleotides are expressed at the apical surface.
The targeting profile of the family of P2Y receptors revealed an unexpected pattern. P2Y receptors activated solely by adenine nucleotides, i.e., the P2Y1, P2Y11, and P2Y12 receptors, are localized to the basolateral membrane of MDCK(II) epithelia, whereas those P2Y receptors activated by uridine nucleotides, i.e., P2Y2, P2Y4, and P2Y6 receptors, are localized to the apical membrane (Fig. 5). The P2Y14 receptor, which is activated by UDP sugars such as UDP-glucose, is also localized to the basolateral membrane. However, the targeting of this receptor may be more a function of its high homology to the adenine nucleotide-selective Gi-coupled P2Y12 receptor than to its ligand. The significance of this unusual localization pattern is unclear, but one intriguing possibility may be that the distribution of P2Y receptors has evolved to complement the preferential release of adenine nucleotides at the basolateral membrane and uridine nucleotides at the apical membrane. However, it is well documented that both ATP and UTP are released from the apical surface of epithelial cells in response to mechanical stimulation and hypotonic challenge (16, 24). In addition, Lazarowski and Harden (23) demonstrated a general release of UTP from primary epithelial cells, although the relative amounts released from the two membrane surfaces is unknown and difficult to measure because of the complex nature of nucleotide metabolism and conversion that occurs within the interstitial space. Thus the significance, if any, of this differential targeting of P2Y receptors remains unclear.

An important question in these studies was whether our targeting data with P2Y receptors in MDCK cells could be extrapolated to epithelial cells from other tissues. For example, it has been shown that the transferrin receptor, which is expressed on the basolateral surface in MDCK(II) cells, is expressed at the apical surface in porcine kidney LLC-PK1 cells (12). This difference in targeting was subsequently attributed to the absence of a protein (the β1b subunit of the adaptor protein AP1) in LLC-PK1 cells (12). To address this concern, we examined the targeting profiles of the Gq-coupled P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 receptors in two other epithelial cell lines: 16HBE14o- cells, which is derived from bronchial epithelial cells transformed with the SV40 virus (7), and Caco-2, which is derived from a colonic adenocarcinoma (11). Our data demonstrated that the polarized expression of the Gq-coupled P2Y receptor subtypes does not depend on the type of epithelial cell in which they are expressed (Fig. 3).

The only receptor that deviated somewhat from its targeting profile obtained in MDCK(II) cells was the P2Y2 receptor, which in addition to its primarily apical localization was also expressed at lower levels along the lateral membranes of 16HBE14o- cells. Interestingly, this low level of lateral staining also was observed in another human epithelial cell line derived from lung, BEAS-2B (37) (data not shown), but not in Caco-2 cells (Fig. 3), suggesting that the small amount of lateral staining of the P2Y2 receptor may be a property of airway cells in particular. Consistent with this observation, Boucher and colleagues (16, 32) demonstrated that UTP promoted intracellular Ca2+ mobilization when added to the basolateral surface of nasal epithelium derived from wild-type mice. These responses were not observed in nasal epithelium derived from P2Y2 receptor (−/−) mice, demonstrating that the responses are due to activation of basolateral P2Y2 receptors. The physiological relevance of this observation is not clear, but our results suggest that the mechanisms utilized by epithelial cells to target the P2Y2 receptor to the apical membrane are not as stringent in epithelial cells from lung compared with those from other tissues.

Although the polarized targeting of the Gq-coupled P2Y receptors is consistent with the majority of results based on functional activity (10, 26, 29, 44), our results conflict with several reports on the polarized expression of P2Y receptors in epithelial cells. For example, Sage and Marcus (39) suggested a basolateral localization for P2Y2 in vestibular dark epithelia on the basis of immunostaining with a commercial antibody. However, results based on commercial P2Y2 receptor antibody staining alone should be viewed with caution, because these antibodies exhibit questionable specificity for P2Y2 receptors.

In conclusion, we utilized three different approaches, including the direct method of confocal microscopy, to show the highly polarized expression pattern of the entire family of P2Y receptors. These data are for the most part consistent with previous reports and extend our knowledge of the localization of P2Y receptors in epithelial cells. Because the polarization of cell surface proteins to either the apical or basolateral membrane of epithelial cells is achieved by the presence of targeting signals within the primary protein sequence, our data suggest that seven of the eight hP2Y receptors contain targeting signals that direct their expression to one of the two membrane surfaces of MDCK cells. Studies to identify these targeting signals in P2Y receptors and to understand how these signals function are currently in progress.

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