ABSTRACT

P2Y12 receptor (P2Y12-R) signaling is mediated through G_i, ultimately reducing cellular cAMP levels. Because cAMP is a central modulator of arginine vasopressin (AVP)-induced water transport in the renal collecting duct (CD), we hypothesized that if expressed in the CD, P2Y12-R may play a role in renal handling of water in health and in nephrogenic diabetes insipidus. We found P2Y12-R mRNA expression in rat kidney, and immuno-localized its protein and aquaporin-2 (AQP2) in CD principal cells. Administration of clopidogrel bisulfate, an irreversible inhibitor of P2Y12-R, significantly increased urine concentration and AQP2 protein in the kidneys of Sprague–Dawley rats. Notably, clopidogrel did not alter urine concentration in Brattleboro rats that lack AVP. Clopidogrel administration also significantly ameliorated lithium-induced polyuria, improved urine concentrating ability and AQP2 protein abundance, and reversed the lithium-induced increase in free-water excretion, without decreasing blood or kidney tissue lithium levels. Clopidogrel administration also augmented the lithium-induced increase in urinary AVP excretion and suppressed the lithium-induced increase in urinary nitrates/nitrites (nitric oxide production) and 8-isoprostane (oxidative stress). Furthermore, selective blockade of P2Y12-R by the reversible antagonist PSB-0739 in primary cultures of rat inner medullary CD cells potentiated the expression of AQP2 and AQP3 mRNA, and cAMP production induced by dDAVP (desmopressin). In conclusion, pharmacologic blockade of renal P2Y12-R increases urinary concentrating ability by augmenting the effect of AVP on the kidney and ameliorates lithium-induced NDI by potentiating the action of AVP on the CD. This strategy may offer a novel and effective therapy for lithium-induced NDI.

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which CD develops AVP resistance in Li-induced NDI is not established. Current therapies for Li-induced NDI are prone to adverse effects. Understanding the mechanism(s) of Li-induced NDI or developing modalities to reverse the AVP resistance of CD will result in new and safer therapies.

Previously we reported that purinergic signaling mediated by ATP/UTP-activated P2Y2 receptor (P2Y2-R) plays a significant role in the genesis of NDI. First, using a rat model, we identified a potential role for P2Y2-R in acquired NDI induced by Li treatment or postobstructive uropathy. P2Y2-R is known to exert tonic inhibitory effect on the AVP-V2-R-AQP2 pathway. Therefore, to further elucidate the role of P2Y2-R, we used mice with genetic deletion of P2Y2-R and found that these mice are significantly resistant to Li-induced NDI. Although promising, currently there are no Food and Drug Administration (FDA)-approved or experimental drugs that can selectively block P2Y2-R in vivo. Hence, we examined the ADP-activated P2Y12-R as another potential P2Y receptor subtype that may oppose AVP-induced water flow in the CD. Phylogenetically P2Y12-R is distinct from P2Y2-R and related receptors. P2Y12-R is expressed predominantly in blood platelets and microglia and astrocytes in the brain. P2Y12-R signaling in platelets is mediated through G, inhibiting adenyl cyclase (AC) and activation of class I phosphoinositol 3-kinase. P2Y12-R is also coupled to G, which through phospholipase C activation increases intracellular Ca needed for platelet activation. Both inhibition of AC activity and activation of phospholipase C are known to decrease the sensitivity of CD to AVP. Hence, we hypothesized that if expressed in the kidney, P2Y12-R may play a role in renal handling of water and in pathophysiology of NDI. The availability of an FDA-approved and time-tested drug, clopidogrel bisulfate, to selectively block P2Y12-R allowed us to test our hypothesis in rat models. Clopidogrel is an oral thienopyridine class of drug that irreversibly inhibits P2Y12-R. It is a prodrug activated in the liver by cytochrome P450 enzymes (e.g., CYP2C19) generating its active metabolite, which constitutes approximately 15% of the ingested drug molecule. In this study we document that P2Y12-R is expressed in the rat kidney, and its pharmacologic blockade increases urine concentrating ability and significantly ameliorates Li-induced NDI in rats.

RESULTS

Expression of P2Y12-R in the Kidney

To immunolocalize P2Y12-R in the kidney, we designed, generated, and characterized a peptide-derived antibody specific for rat or mouse P2Y12-R (Supplemental Table 1). We evaluated the specificity of our antibody by immunoblotting and immunofluorescence (IF) microscopy in two different cell lines, namely inner medullary collecting duct-3 (IMCD3) (mouse renal CDs) and HEK293T (human embryonic kidney), and by using two different approaches: P2Y12-R gene knockdown and overexpression (Figure 1). For proper

Figure 1. Characterization of specificity of P2Y12-R antibody. (A) Western analysis of knockdown (KD) of P2Y12-R showing receptor protein abundance in IMCD3 cells transfected with scrambled (CT) or P2Y12-R specific shRNA (KD), relative to the respective protein abundances of β-actin; the table below the blots shows the percent P2Y12-R protein remaining after KD. (B) IF detection of GFP (green) and P2Y12-R (red) in the IMCD3 cells transfected with shRNA plasmids specific for P2Y12-R. Cell nuclei were stained with propidium iodide (blue). Upper panel shows P2Y12-R labeling in a profile of cells, whereas the lower panel is overlay of GFP labeling on the same profile of cells. Note a near complete KD of P2Y12-R expression (arrows in the upper panel) in transfected cells (arrows in the lower panel). Asterisks indicate nontransfected cells showing P2Y12-R plasmids specific for P2Y12-R. Cell nuclei were stained with propidium iodide (blue). Upper panel shows P2Y12-R labeling in a profile of cells, whereas the lower panel is overlay of GFP labeling on the same profile of cells. Note a near complete KD of P2Y12-R expression (arrows in the upper panel) in transfected cells (arrows in the lower panel). Asterisks indicate nontransfected cells showing P2Y12-R labeling (CT) or human P2Y12-R overexpressing (OE) HEK293T cells relative to the respective protein abundances of β-actin; the table below the blots shows the percent overexpression of P2Y12-R protein. Act., actin.
interpretation of the data presented in Figure 1, one needs to take into account that P2Y₁₂-R exists in multiple forms (monomer, dimer, trimer, or oligomer), with each form in turn having different glycosylated subspecies, spanning from 35 to >220 kD in immunoblots. Furthermore, the expression of these multiple forms and glycosylated subspecies may differ depending on the nature of the cells under consideration. As shown in Figure 1A, the control IMCD3 cells showed a major band at approximately 80 kD and a faint band at approximately 60 kD, which match with the same size bands seen in rat kidney and brain (Figure 2B). In IMCD3 cells subjected to knockdown of P2Y₁₂-R by shRNA (KD), there was an approximate 55% decrease in relative intensity of the 80 kD band and disappearance of 60 kD band. In parallel, IF imaging of IMCD3 cells showed labeling for P2Y₁₂-R (red) only in green fluorescent protein (GFP)-negative untransfected cells (asterisks), but not in GFP-positive transfected cells (arrows) (Figure 1B). As shown in Figure 1C, in control HEK293T cells, our antibody recognized a clear band at approximately 60 kD, which matches with the same size band in IMCD3 cells (Figure 1A). In addition, we could see a faint band at approximately 38 kD, which matches with the same size band in the native rat kidney (Figure 2B). In cells over expressing P2Y₁₂-R, both bands showed a marked increase in intensity, associated by the appearance of a new band at approximately 55 kD, which matches with the similar size band in human platelet lysates when probed with our P2Y₁₂-R antibody (Supplemental Figure 1). Therefore, taken together, the data presented in Figure 1 validated the specificity of our P2Y₁₂-R antibody. Because manipulation of P2Y₁₂-R protein levels in the cells by knockdown or overexpression at the gene level matched with corresponding changes in the intensity of all of the detected bands, we can conclude that all detected bands are specific to P2Y₁₂-R. Further validation of our P2Y₁₂-R antibody was achieved by IF labeling of mouse microglial cells (Supplemental Figure 2).

Figure 2B shows identical P2Y₁₂-R protein bands in rat kidney and brain, which are ablated by the blocking peptide and could not be visualized when the blot was probed with preimmune IgG fraction. In the kidney samples our antibody identified two bands (one weak band at 38–40 kD, another strong band at 80 kD) in the cortex and outer medulla and one weak band (approximately 80 kD) in the inner medulla. On the basis of the predicted molecular weight of the P2Y₁₂-R protein (38 kD; 347 amino acid residues), it appears that the weak band at 38–40 kD is the monomeric form of the receptor protein, whereas the strong band at 80 kD represents a dimeric form. Real-time RT-PCR detected P2Y₁₂-R mRNA in the renal cortex and outer and inner medullas of Sprague–Dawley rats (Figure 2A).

**Cellular Localization of P2Y₁₂-R in the Rat Kidney**

Immunoperoxidase reaction revealed strong labeling for P2Y₁₂-R on the brush border of the proximal tubule in the cortex (Figure 3A), which was ablated by peptide block and was not seen when probed with preimmune IgG (Figure 3, B and C). Weak labeling was found at the apical domain of the CD in the medulla and cortex (Figure 3, D and E). Confocal IF imaging confirmed expression of P2Y₁₂-R over the brush border of proximal tubules, revealed labeling in arterioles (Figure 3F), and showed P2Y₁₂-R protein in AQP2 positive cells in the cortex (Figure 3, G) and medulla (Figure 3, H–K), with an apparent localization of both proteins on the apical membrane of the CD cells (Figure 3, G, H, and K). Similar observations were made in IF when P2Y₁₂-R antibody from Alomone Labs was used (Supplemental Figure 3).

**Administration of Clopidogrel Ameliorated Li-Induced NDI**

Administration of clopidogrel significantly ameliorated polydipsia, polyuria, and decrease in urine osmolality (Figure 4, A–C) and reversed the increase in electrolyte-free water excretion (Figure 4D) induced by Li feeding to rats for 2 weeks. These were associated with significant improvement in Li-induced decrease in AQP2 protein in the inner medulla (Figure 4, E and F). IF labeling for AQP2 protein in the inner medullas of control rats was predominantly on the apical aspect of the medullary CD cells (Figure 5A). In Li-treated rat kidneys the AQP2 labeling in the medullary CD was scanty (Figure 5B). After clopidogrel administration, AQP2 labeling was seen all over the cells, with relatively higher intensity on the apical domain (Figure 5C). In rats coadministered with Li and clopidogrel, the intensity of AQP2 labeling improved with predominant apical labeling (Figure 5D).
Clopidogrel administration augmented Li-induced increase in urinary AVP excretion (Figure 6A). Administration of clopidogrel had no significant effect on Li-induced natriuresis (Figure 6B), but effectively reversed the significant increases in urinary total nitrates/nitrites, an index of production of nitric oxide (Figure 6C), and 8-isoprostane, a measure of oxidative stress (Figure 6D). Finally, clopidogrel did not decrease lithium levels in the blood (Figure 7A) or in the inner medulla (Figure 7B).

**Blockade of P2Y12-R Enhanced the Effect of Desmopressin in Rat IMCD**

Interestingly, clopidogrel administration *per se* significantly increased urinary excretion of AVP (0.54±0.09 in controls versus 1.23±0.18 in clopidogrel treated, in µmol/24 h per 200 g body wt, n=4/group, P<0.01). Preliminary studies revealed expression of P2Y12-R mRNA in rat hypothalamus and significant increase in AVP mRNA expression by selective blockade of P2Y12-R by PSB-0739 in cultured rat hypothalamic cells (Supplemental Figure 4). These findings suggest that the observed increase in urinary AVP excretion in clopidogrel-treated rats was apparently mediated through the blockade of P2Y12-R in hypothalamus. In line with this, in the clopidogrel alone treated group, there was a significant increase in AQP2 protein abundances in the renal cortex and inner medulla (Supplemental Figure 5). These effects were apparently mediated by an increase in AVP levels and by potentiation of AVP effects on the CD by the blockade of P2Y12-R (subsequently discussed). However, the effects of clopidogrel were not mediated through UTP/ATP-activated P2Y2-R, because when administered to P2Y2-R knockout and wild-type mice, clopidogrel had identical effects on urinary concentration (Supplemental Figure 6).

To investigate whether blockade of P2Y12-R had a direct effect on the CD, we performed experiments on primary cultures of rat IMCD cells, where we clamped the concentration of desmopressin (dDAVP; a V2 receptor specific analog of AVP) and used PSB-0739, a selective and reversible antagonist of P2Y12-R. Unlike clopidogrel, PSB-0739 is not a prodrug and therefore is suitable for cell cultures. Blockade of P2Y12-R with PSB-0739 significantly increased dDAVP-induced AQP2 expression by 3.4-fold, but PSB-0739 alone had no effect on AQP2 expression, and enhanced the dDAVP-induced AQP3 expression (Figure 8). PSB-0739 did not augment the increase in dDAVP-induced expression of V2 receptor. However, combination of dDAVP and PSB-0739 significantly increased V2 receptor expression. In parallel cultures, we observed that PSB-0739 significantly enhanced the dDAVP-induced cAMP production (Figure 8). Taken together, these data suggest that blockade of P2Y12-R in IMCD in the absence of dDAVP had little or no effect, but it markedly potentiated the effect of dDAVP on AQP2 and AQP3 expression, and on cAMP production. Interestingly, the lack of effect of PSB-0739 alone in CD cells is further supported by our observation that clopidogrel *per se* had no effect on the urinary concentration in Brattleboro rats that lack AVP (Supplemental Figure 7). Therefore, the observed effect of blockade of P2Y12-R on urinary concentration in Sprague–Dawley rats is dependent on AVP.

**DISCUSSION**

We demonstrated that (1) P2Y12-R is expressed in rat kidney and is localized in the CD cells; (2) administration of
clopidogrel, a selective and irreversible antagonist of P2Y12-R ameliorated Li-induced NDI in rats, without decreasing Li levels in the blood or in the renal inner medulla; and (3) selective blockade of P2Y12-R by PSB-0739 in primary cultures of rat IMCD cells potentiated the expression of AQP2 and AQP3 and cAMP production induced by dDA VP. In parallel, we documented that administration of clopidogrel alone increased urinary concentrating ability and AQP2 protein abundance in rat kidney in an AVP-dependent manner. Our observations define a new role of P2Y12-R in the kidney and suggest that pharmacologic blockade of P2Y12-R per se has marked effect on CD and the observed in vivo effect of clopidogrel in rats is not just caused by increased levels of AVP. The latter is in turn supported by the improved abundance and cellular disposition of AQP2 protein in the renal medulla and CD, respectively. It is also clear that the ameliorating effect of clopidogrel was not caused by impaired absorption of Li in the intestines, resulting in lower blood Li levels or reduced accumulation of Li in the renal medulla. Although administration of clopidogrel augmented Li induced increase in urinary AVP excretion, one cannot attribute this effect of clopidogrel per se to its ability to ameliorate Li-induced NDI because Li-induced NDI is caused by the resistance of the kidney to circulating AVP levels, which are often high. Because we showed that in primary cultures of rat IMCD cells blockade

Figure 4. Effect of clopidogrel administration on Li-induced polyuria and decrease in AQP2 protein abundance in rats. (A) Water intake; (B) urine output; (C) urine osmolality; and (D) electrolyte-free water clearance on the last day (day 13). CNT, control group; CLPD, clopidogrel-treated group; LI, Li-treated group; LI+CLPD, combined treatment with Li and clopidogrel. n=7 rats/group for (A), (B), and (C); n=4–5 rats for (D). *P<0.05 versus Li groups by ANOVA followed by Tukey–Kramer multiple comparison test [(A)–(D)]. (E) Representative immunoblot of AQP2 and β-actin proteins run with inner medullary tissue samples. (F) Densitometry of AQP2 protein bands in Li and Li+CLPD groups relative to β-actin protein bands; P value by Mann–Whitney nonparametric method.

dDA VP is markedly enhanced. The subdued activity of phospholipase C during the blockade of P2Y12-R may also contribute to the potentiation of AVP/dDA VP effect on CD. However, in the absence of AVP, PSB-0739 alone has no effect on AQP2 gene expression in the cultured IMCD cells, which is consistent with our finding of lack of effect of clopidogrel in Brattleboro mutant rats.

Clopidogrel not only prevented Li-induced polyuria but reversed the significant increase in electrolyte-free water excretion, which suggest that clopidogrel is acting on CD. The latter is in turn supported by the improved abundance and cellular disposition of AQP2 protein in the renal medulla and CD, respectively. It is also clear that the ameliorating effect of clopidogrel was not caused by impaired absorption of Li in the intestines, resulting in lower blood Li levels or reduced accumulation of Li in the renal medulla. Although administration of clopidogrel augmented Li induced increase in urinary AVP excretion, one cannot attribute this effect of clopidogrel per se to its ability to ameliorate Li-induced NDI because Li-induced NDI is caused by the resistance of the kidney to circulating AVP levels, which are often high. Because we showed that in primary cultures of rat IMCD cells blockade
of P2Y12-R potentiates dDAVP effect on AQP2 water channel expression and cAMP production, it is reasonable to suggest that blockade of P2Y12-R by clopidogrel might have sensitized the CD during Li treatment and therefore relieved the NDI.

In addition to its effect on the CD, the blockade of P2Y12-R had other beneficial effects on the kidney that might contribute to amelioration of Li-induced NDI. These are the reversal of Li-induced increase in nitric oxide production and oxidative stress. Endogenously derived nitric oxide exerts tonic inhibition on the expression of sodium transporters, which in turn may contribute to impaired urinary concentrating ability in Li-induced NDI. Oxidative stress is known to repress expression of several genes, including transcription factors, however, less is known about its significance in relation to renal transport functions.

The significance of predominant expression of P2Y12-R on the brush border of proximal tubules is not known at this stage. However, P2Y12-R does not seem to have an effect on the bulk absorption of sodium because urinary sodium levels were not altered by the administration of clopidogrel alone or in combination with Li. A parallel study conducted in a mouse model, where we analyzed the sodium transporter/channel/exchanger profile, also did not support a major role for the proximal tubules.

Li-induced NDI is associated with CD remodeling, with a decreased number of principal cells and an increased number of intercalated cells. Hence, there is a possibility that the observed effect of clopidogrel administration on Li-induced decrease in AQP2 protein may be related to its ability to interfere in CD remodeling. However, the IF data in clopidogrel- and/or Li-treated rats do not suggest that possibility in our short-term model.

Finally, previous reports showed that P2Y12-R may contribute to early mesangial cell transformation and renal vessel hypertrophy or altered renal autoregulation, both in the setting of angiotensin II–induced hypertension. Recent studies by Morel et al. also demonstrated an impaired platelet P2Y12-R inhibition in CKD, which appears to be related to the effect of pathophysiologic milieu of CKD on platelet activity. Furthermore, most clinical studies on clopidogrel bisulfate were designed with the criteria of evaluation on the basis of cardio- or cerebrovascular events, but not renal effects, and therefore were prone to miss the findings documented in our study.

**CONCISE METHODS**

**Animal Experiments**

Animal procedures were approved by the Institutional Animal Care and Use Committees of the VA Salt Lake City Health Care System and the University of Utah. Male Sprague–Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). Brattleboro mutant rats that lack AVP (HsdBlu:BRAT-Avpdi) were obtained from the Rat Resource & Research Center, University of Missouri (Columbia, MO). All animals had free access to drinking water and standard food during the experimental period. NDI was induced by feeding Li-added food (40 mmol LiCl/kg food; MP Biomedicals, Solon, OH) to rats for 13 days. Clopidogrel bisulfate was administered orally by mixing finely powdered clopidogrel bisulfate tablets in drinking water. The concentration of the drug in the drinking water was adjusted daily on the basis of water consumption of the animals on the previous day. Clopidogrel bisulfate was administered orally by mixing finely powdered clopidogrel bisulfate tablets (Bristol-Myers Squibb, New York, NY) in drinking water. The concentration of the drug in the drinking water was adjusted daily on the basis of water consumption of the animals on the previous day. Sprague–Dawley rats received 20 mg clopidogrel/kg body wt per day, whereas the Brattleboro rats

**Figure 5.** Effect of clopidogrel treatment on Li-induced cellular expression and disposition of AQP2 (green) and P2Y12-R (red) proteins in rat IMCD. Representative low magnification profiles of IF labeling for AQP2 and P2Y12-R in the renal medullas of rats treated with no drug (A), Li (B), clopidogrel (C), or a combination of Li and clopidogrel (D). Insets show corresponding higher magnification profiles. Bar 20 μm.

**Figure 6.** Effect of clopidogrel on Li-induced alterations in urinary parameters. (A) Urine AVP; (B) urine sodium; (C) urine total nitrates (NO3) and nitrites (NO2); and (D) urine 8-isoprostane. bw, body weight; CNT, control group; CLPD, clopidogrel group; Li, Li alone; Li+CLPD, combination of Li and CLPD. n=4–6 rats per group. *Significantly different from all other groups by ANOVA followed by Tukey–Kramer multiple comparison test.
received double this dose (40 mg/kg body wt per day). The rationale for the doses of clopidogrel used is provided in the Supplemental Material.

Twenty-four hour urine samples were collected by placing the animals in plastic metabolic cages (1 rat per cage). Blood and kidney tissues were collected at necropsy. Cortical, outer, and inner medullary regions of the kidneys were dissected out and processed for laboratory analysis. In some untreated Sprague–Dawley rats, samples of cerebral cortex and whole hypothalamic tissue were collected and processed.

**Synthesis and Characterization of PSB-0739**

PSB-0739 (1-amino-4-[4-phenylamino-3-sulfophenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate) is a highly potent, selective, reversible non-nucleotide antagonist of P2Y12-R that is not toxic to the cells. Unlike clopidogrel, PSB-0739 does not require bioactivation; therefore, it is suitable for use in cell cultures. PSB-0739 was designed, synthesized, purified, and characterized in the laboratory of Dr. Christa Müller at the University of Bonn, Bonn, Germany.

**Blood, Urine, and Tissue Analysis**

Osmolalities of clear urine samples were measured by vapor pressure method (Wescor, Logan, UT). Sodium, potassium, and Li levels in serum and/or urine were determined on EasyElectrolytes (Medica, Bedford, MA). On the basis of serum and urine analysis, electrolyte-free water clearance $[\text{CH}_2\text{O}(\text{e})]$ was computed using the formula $\text{CH}_2\text{O}(\text{e})=V(1-\text{UNa+UK})/P\text{Na}$, where $V$=urine volume, $\text{UNa}$ and $\text{UK}$ are urine Na and K concentrations, and $P\text{Na}$ is plasma Na concentration, respectively. Li levels in the inner medullary tissue homogenates were assayed by inductively coupled plasma-mass spectrometry (Exova, Santa Fe Springs, CA) and were normalized to their respective protein contents. Inductively coupled plasma-mass spectrometry has a detection limit of 0.06 parts per million for Li, with recovery of 91% in spiked samples. Li was not detectable in the homogenization medium (0.5% SDS and 0.02% sodium azide in ultrapure water). Commercial enzyme immunoassay/ELISA or colorimetric assay kits were used to quantify urinary excretion of AVP (Enzo Life Sciences, Farmingdale, NY), total nitrates and nitrites (NO$_3$/NO$_2$) (Cayman Chemical, Ann Arbor, MI), and 8-isoprostane (Oxford Biomedical Research, Oxford, MI) as per the manufacturers’ instructions and published in our previous study.

**Detection of mRNA by Real-Time RT-PCR Assay**

This was performed essentially as described previously. Brieﬂy, total RNA was extracted from samples of kidney cortex, outer and inner medullas, cerebral cortex, and whole hypothalamus. cDNA was synthesized by SuperScript Reverse transcription (Invitrogen, Carlsbad,
CA). Using gene-specific primers (Supplemental Table 2), real-time PCR amplifications were carried out on cDNA samples in Applied Biosystems 7500 Real-Time PCR System (Foster City, CA), with AmpliTaq Gold and SYBR Green for detection. Expression of target genes was normalized to that of the housekeeping gene β-actin.

**Generation and Characterization of P2Y12-R Antibody**

We designed and generated a peptide-derived rabbit polyclonal antibody for a 16-amino acid C-terminus sequence (KKKGQEGGEPSEETPM) of mouse P2Y12-R (accession no. Q9CPV9), which differs from the corresponding rat sequence by one amino acid residue. This peptide has no sequence homology with related proteins, such as P2Y1a, P2Y1b, GPR34, GPR82, GPR87, or GPR171 (Supplemental Table 1). Peptide synthesis, purification, and coupling to keyhole limpet hemocyanin, immunization of the rabbits, and determination of antibody titers by ELISA were contracted to thermo Fisher Scientific (Rockford, IL). Affinity purification of the antibody and isolation of preimmune IgG fractions and characterization of the specificity of the antibody were done in our laboratories as described previously.36,37

**P2Y12-R Knockdown and Overexpression**

The specificity of the antibody was evaluated using knockdown and overexpression approaches in cells. IMCD3 cells, a mouse inner medullary CD cell line (ATCC, Manassas, VA), were maintained in a 1:1 mixture of dulbecco’s modified Eagle’s medium and F-12 (Life Technologies, Grand Island, NY), supplemented with 10% FBS (Life Technologies) at 37°C with 5% CO2. For transfection via electroporation, IMCD3 cells were grown to 80%–90% confluency, trypsinized, and resuspended in warm complete medium. Cells were pelleted, suspended in Nucleofector solution (Lonza Cell Transfection System), and transfected with mouse P2ry12 SureSilencing shRNA, GFP plasmids (Qiagen, Valencia, CA). DNA from the four individual P2Y12 shRNA plasmids (1.5 µg of each plasmid, totaling 6 µg) were mixed with the suspended cells (0.5 × 10⁴ cells). Cells were transfected using the program A24 on the transfection device (Lonza Amax Biosystems Nucleofector I). Each transfection was plated in an individual well of a 12-well plate. Two days after transfection, the cells were processed for either immunoblotting or IF microscopy. For immunoblotting, cells were washed with PBS and collected into 1× blue loading buffer containing DTT (Cell Signaling, Danvers, MA). Lysed cells were homogenized using Tissuelyser (Qiagen) for 10 minutes and heated at 95°C for 5 minutes. The samples were processed for Western blotting. For IF microscopy, cells growing on chamber slides were washed briefly with PBS and then fixed in 2% paraformaldehyde for 20 minutes and permeabilized in 0.2% Triton X-100. P2Y12-R antibody (which we generated) was used in combination with Cy5 anti-rabbit secondary antibody (Thermo Fisher Scientific, Pittsburgh, PA) to detect the receptor protein in the transfected IMCD3 cells. Propidium iodide (Sigma-Aldrich, St. Louis, MO) was used to stain the nuclei. Cells were visualized in Personal Confocal Microscope PCM-2000 (Nikon).

In another experiment, lysates of HEK293 T cells overexpressing human P2Y12-R and control lysates were purchased from the Novus Biologicals. The overexpression cells were created using plasmid ID RC205005 and on the basis of accession number NM_022788. The cells were lysed in modified RIP buffer containing protease cocktail mixture by the Novus and were shipped to us. The control and over-expressing cell lysates were processed for immunoblotting using our P2Y12-R antibody.

**Immunoblotting**

Processing of the kidney tissue samples and immunoblotting were performed as described previously.13,16 Negative controls for P2Y12-R antibody were generated by preadsorbing the affinity purified antibody with a molar excess of the immunizing peptide or by substituting the antibody with preimmune IgG fraction. Generation and characterization of rabbit polyclonal antibody (GN-762) to AQP2 was described previously.37 Rabbit polyclonal antibody to AQP1 was a kind gift from Dr. Janet Klein (Emory University, Atlanta, GA), and β-actin rabbit polyclonal antibody was purchased from Biolegend (San Diego, CA). Where applicable, protein band densities were determined, and differences in protein loading were corrected by normalizing the band densities of the target proteins to that of β-actin.

**Localization of P2Y12-R Protein by Immunoperoxidase Labeling**

Formalin-fixed and paraffin-embedded kidney sections (5 µm) were processed for immunoperoxidase labeling as described previously,36 except for the following modifications. Dako target retrieval solution (Dako North America, Carpenteria, CA) was used for antigen retrieval. Negative controls for labeling were generated by incubating the sections with P2Y12-R antibody preadsorbed with a molar excess of the immunizing peptide or preimmune IgG fraction in lieu of the affinity purified P2Y12-R antibody. Sections were counterstained with methyl green.

**Localization of P2Y12-R with AQP2 Protein by Confocal IF**

This was performed essentially as described previously.36 Sections were probed with P2Y12-R rabbit polyclonal antibody overnight followed by incubation with secondary Alexa Fluor 594-conjugated goat anti-rabbit antibody (Invitrogen) for 1 hour. Some sections were double labeled first by incubation with AQP2 mouse monoclonal antibody, followed by incubation for 1 hour with goat anti-mouse secondary Alexa Fluor 488-conjugated antibody. DAPI (4’,6-diamino-2-phenylindole) was used to stain the nuclei.

**Primary Cultures of Rat Inner Medullary CD Cells**

Primary cultures of rat IMCD cells were prepared as described previously38 with the following modifications. Freshly obtained inner medullas were cut into small pieces and digested in PBS containing 1 mg/ml collagenase B (Roche Applied Sciences, Indianapolis, IN) and 0.1 mg/ml DNase (Sigma-Aldrich) at 37°C for 1 hour. After that, non-IMCD cells were eliminated by osmotic shock by adding two volumes of sterile water. The suspension was filtered through 100-µm cell strainer and centrifuged at 1000 rpm for 5 minutes. The pellet was washed with 10% BSA in PBS, suspended in modified defined medium (DMEM/Ham’s F12; composition given in the Supplemental Material) containing 10% FBS, and seeded into Transwell semipermeable support inserts (Corning Costar, Cambridge, MA). Cells were incubated at 37°C in an atmosphere of 5% CO2. The medium was replaced every 2 days, and when the cells became 70%–80% confluent, the
medium was changed to the starvation one (composition given in the Supplemental Material). After 24 hours the test agents, namely dDAVP (desmopressin; Sigma-Aldrich) and/or PSB-0739, were added, and the incubation continued for another 48 hours. After removing the medium, cells were collected into Trizol reagent for total RNA extraction. For cAMP assay, the medium was gently aspirated an 200 μl of 0.1 M HCl was added to each insert and incubated for 20 minutes at room temperature. Then cells were scrapped off the semipermeable support and lysed by pipetting up and down. The cell lysate was centrifuged at 1000 g for 10 minutes. cAMP concentration in the supernatant was determined by an ELISA Kit (Cayman Chemical) and normalized to the protein content (Pierce BCA Protein Assay Kit; Rockford, IL).

Statistical Analyses

All quantitative data were expressed as mean±SEM. Comparisons among the means of multiple groups were made by ANOVA, followed by the assessment of statistical significance by Tukey–Kramer multiple comparison test or Student–Newman–Keuls multiple comparison test of Bonferroni test. Differences between the means of two groups were determined by unpaired t test or where applicable, by Mann–Whitney non-parametric test. P values <0.05 were considered significant. GraphPad Instat (GraphPad Software, La Jolla, CA) was used for statistical analyses.

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DISCLOSURES

The use of P2Y12 receptor selective antagonists for the treatment of NDI is proprietary to the US Department of Veterans Affairs and the University of Utah Research Foundation and is protected by a pending patent application with the US Patent and Trademark Office.

REFERENCES


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