Potential role of purinergic signaling in lithium-induced nephrogenic diabetes insipidus

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Submitted 30 December 2008; accepted in final form 20 February 2009

LITHIUM (Li) has been in clinical use for about half a century, mostly for the treatment of bipolar disorder, which affects up to 2% of the US population (33), with double the incidence in male veterans. Mental depression and substance abuse, which are often encountered in posttraumatic stress disorder patients, as well as chronic neurodegenerative diseases, such as Alzheimer, Parkinson, and Huntington diseases (12, 35, 45, 53)

WITH Li (38). Furthermore, recent studies unraveled the protective effect of Li, thus making it a potential drug for the treatment of acute brain injury (e.g., stroke or ischemia), as well as chronic neurodegenerative diseases, such as Alzheimer, Parkinson, and Huntington diseases (12, 35, 45, 53).

Whether it is for bipolar disorder or chronic neurodegenerative diseases, Li therapy is administered for long durations. However, therapeutic use of Li is often limited because of its adverse side effects on the kidney. In the clinic, patients on Li therapy often present with classical symptoms of nephrogenic diabetes insipidus (NDI), which include polyuria, polydipsia, and reduced ability to concentrate urine and a lack of response to arginine vasopressin (AVP) administration (18). In animal models, Li-induced polyuria is associated with decreased protein abundance of AVP-regulated collecting duct water channel aquaporin (AQP)2 (31, 36). The AVP resistance in Li-induced NDI has been attributed to increased production of renal prostaglandin (PG)E2 (48). PGE2 is a potent antagonist of AVP action on collecting duct (19, 40, 44, 56). Administration of indomethacin, which inhibits PGE2 biosynthesis, ameliorates the polyuric condition, thus confirming the involvement of PGE2 in the Li-induced polyuria (2).

NDI is a debilitating condition that can result in severe fluid and electrolyte imbalance, especially in elderly patients, who exhibit impaired thirst mechanism (29, 34). Dehydrated NDI patients can develop hypernatremia, alterations in the level of consciousness, and hemodynamic instability from hypovolemia. Currently used therapeutic modalities, such as the combined use of a thiazide with a potassium-sparing diuretic (amiloride) or prostaglandin synthesis inhibitor (indomethacin or other cyclooxygenase inhibitors), are encountered with varying degrees of success as well as side effects, including Li intoxication (39, 41). Replacement of the current side effect-prone therapies with new drugs based on an improved understanding of molecular pathophysiology of Li-induced NDI should result in improved efficacy and fewer side effects.

One potential approach for the development of new therapies could be to decrease the production of renal PGE2, if we can identify the driving force(s) for the increased production of PGE2 by medullary collecting ducts in Li-induced NDI. In this context, we previously identified (54) that extracellular nucleotides (ATP/UTP), presumably acting through purinergic P2y2 receptor in normal rat medullary collecting duct, induce production and release of PGE2. We also reported (49) that this phenomenon is markedly enhanced in sucrose water-induced polyuria and blunted in the dehydrated condition. Hence, we hypothesized that purinergic signaling may play a key role in...
the pathogenesis of Li-induced NDI by enhancing the production of PGE2 by medullary collecting ducts and thus leading to a vasopressin-resistant state. In this study, using multiple approaches that involve ex vivo nucleotide-stimulated production of PGE2 by medullary collecting ducts, determination of expression of mRNA of P2 receptors in the inner medulla by quantitative RT-PCR, and confocal laser immunofluorescence localization of P2y2 and P2y4 receptor proteins in the inner medulla, we document that enhanced purinergic signaling in medullary collecting duct of Li-fed rats may potentially contribute to NDI.

METHODS

Experimental animals. The animal protocols involved in these studies were approved by the Institutional Animal Care and Use Committees of the Department of Veterans Affairs Salt Lake City Health Care System and the University of Utah. Specific pathogen-free male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA).

Li-induced NDI model. Groups of rats were fed either a normal rat chow (control diet) or normal rat chow with added Li chloride (40 mmol/kg chow) with free access to drinking water for 14 or 21 days. Both diets were custom prepared by MP Biomedicals (Aurora, OH). Twenty-four-hour urine output and water intake were monitored before the experimental period, periodically during the Li feeding, and before euthanasia. At the end of the experimental period rats were euthanized by an i.p. injection of sodium pentobarbital, and kidney tissue samples were collected for analysis. Serum levels of Li were assayed on an automated clinical chemistry analyzer. Urine osmolality was measured by vapor pressure method on an osmometer (Wescor, Logan, UT). About 40 rats (n = 20 each for control and Li-added diet) were used in this study.

Determination of urinary PGE2 excretion. PGE2 is not stable in the urine. It is rapidly converted to its 13,14-dihydro-15-keto metabolite. Hence, using a Prostaglandin E Metabolite Kit (Cayman Chemical, Ann Arbor, MI), we converted urinary PGE2 to a stable derivable and quantified it by enzyme immunoassay (EIA) as described previously (49, 50). The PGE2 metabolite content of the urine is expressed as nanograms per day or nanograms per milligram of creatinine (n = 8 urine samples each for control and Li-added diet groups). Creatinine was assayed by using a Creatinine Assay Kit from BioAssay Systems (Hayward, CA).

Measurement of nucleotide-stimulated release of PGE2 by medullary collecting ducts. Fractions enriched in medullary collecting ducts were prepared and assayed for nucleotide-stimulated PGE2 release by the methods established in our laboratories (49, 50, 54). Briefly, freshly obtained inner medullas from control or Li-added diet-fed rats (n = 5 rats in each group) were digested by collagenase and hyaluronidase, followed by low-speed centrifugation and washings to separate the inner medullary collecting ducts from non-inner medullary collecting duct elements. The inner medullary collecting duct suspensions thus obtained were challenged with nucleotides. PGE2 released into the medium was measured by using an EIA kit (Cayman Chemical) after the medullary collecting ducts were pelleted by centrifugation. The values obtained for PGE2 in the incubations were normalized to the protein contents of the respective pellets. Protein was quantified by using a Coomassie Plus Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL).

Quantitative real-time RT-PCR assays on inner medullary samples. This was performed as described previously with minor modifications (28, 50). Briefly, total RNA from the inner medullas of control or Li-added diet-fed rats (n = 4 rats in each group) was isolated by the TRIzol method (Invitrogen, Carlsbad, CA), according to manufacturer’s recommendations. Traces of genomic DNA in the extracted RNA samples were digested by DNase I treatment (DNase I, amplification grade, Invitrogen), followed by the inactivation of DNase by EDTA. cDNA was synthesized by reverse transcription with SuperScript III First-Strand Synthesis SuperMix (Invitrogen). cDNA was quantified by real-time amplifications on the Applied Biosystems 7500 Fast Real-Time PCR System (Foster City, CA) with SYBR Green PCR Master Mix and AmpliTag Gold. Table 1 shows the nucleotide sequences of the primer pairs used in PCR. The DNA was amplified for 40 cycles by setting the annealing temperature to 60°C. Specificity of amplifications was determined by sequencing PCR products. The expression of P2y1, P2y2, P2y4, and P2y6 receptors was computed relative to the expression of the housekeeping gene β-actin.

Immunofluorescence confocal microscopy of inner medullas. This was performed per the methods established in our laboratories (11, 21). Briefly, formalin-fixed and paraffin-embedded kidney sections (20-μm thickness) from control or Li-added diet-fed rats (n = 4 rats in each group) were deparaffinized, rehydrated, and treated with Dako Antigen Retrieval reagent (Dako Cytomation, Carpinteria, CA). The sections were then permeabilized by Triton X-100 treatment, followed by incubation with Image-iT FX Signal Enhancer (Molecular Probes, Carlsbad, CA). Rabbit polyclonal antibodies to P2y2 or P2y4 were used in conjunction with Cy5-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). P2y2 receptor antibody was raised and characterized by us (27, 28). It is directed to a COOH-terminal peptide sequence. P2y4 receptor antibody was purchased from Novus Biological (Littleton, CO). According to the manufacturer, it was raised against the first cytoplasmic domain of human P2y4 receptor, which has 67% rat identity and 80% rat homology. Nuclei were stained with propidium iodide. Confocal laser microscopy was performed with a Nikon E800 upright microscope with Pan Fluor oil immersion lenses. A Simple Personal Confocal Image program (PCI, Compix, Cranberry Township, PA) was used to acquire digital images. Cy5 was detected with a red HeNe laser at 633 nm.

Table 1. Nucleotide sequences of primer pairs used in PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Primer Position</th>
<th>Primer Sequence</th>
<th>Amplicon Size, bp</th>
</tr>
</thead>
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<tr>
<td>P2y1</td>
<td>NM_012800</td>
<td>1235–1254</td>
<td>ACGTGAGAGATGAGTACCTGCG</td>
<td>289</td>
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<tr>
<td></td>
<td></td>
<td>1504–1523</td>
<td>CCCCCCTGGTCTGAAACATAACAC</td>
<td></td>
</tr>
<tr>
<td>P2y2</td>
<td>NM_017255</td>
<td>1270–1293</td>
<td>ACCCGACACCCCTCATTACTCCTTC</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1376–1399</td>
<td>TGGAGAAGACACAGGTCAGGACAC</td>
<td></td>
</tr>
<tr>
<td>P2y4</td>
<td>NM_031680</td>
<td>263–284</td>
<td>TGGTTGACCTGGCATTGTCAG</td>
<td>294</td>
</tr>
<tr>
<td></td>
<td></td>
<td>537–556</td>
<td>AAGATTTGGCAGGCGGGGACGAGCAG</td>
<td></td>
</tr>
<tr>
<td>P2y6</td>
<td>NM_057124</td>
<td>644–665</td>
<td>TGGCTTGGTGTATTGTGGGATGTC</td>
<td>339</td>
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<tr>
<td></td>
<td></td>
<td>960–982</td>
<td>TGGAAAGCGAGGAACGTTGTAAAC</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>NM_031144.2</td>
<td>18–37</td>
<td>CACCCGGGAGTACAACCCTTC</td>
<td>207</td>
</tr>
<tr>
<td></td>
<td></td>
<td>205–224</td>
<td>CCCATACCCAGCATTACAGCC</td>
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</table>

P2y2 receptor primer sequences are as published by us previously (28, 50). Primer pairs for other P2y receptors were based on the sequences published by Wildman et al. (55).
Statistical analysis. Quantitative data are expressed as means ± SE. Differences between the means of two groups were analyzed by unpaired t-test. Differences among the means of more than two groups were analyzed by analysis of variance (ANOVA), followed by the assessment of differences by Tukey-Kramer multiple-comparison test. *P values < 0.05 were considered significant.

RESULTS

Characterization of rat model of Li-induced NDI. We have chosen a diet containing 40 mmol of Li chloride per kilogram of food because this concentration of Li when fed to rats up to 4 wk was shown to induce NDI in animals, mimicking the clinical condition seen in patients, without causing overt Li toxicity (31). Figure 1 shows the characterization of this Li model with respect to polydipsia and polyuria. Compared with the rats on regular diet, Li-fed rats showed 1) statistically significant smaller increases in body weight (Fig. 1A); 2) approximately twofold increase in water consumption (Fig. 1B); 3) two- to threefold increase in urine output (Fig. 1C); and a comparable threefold decrease in urine osmolality (Fig. 1D) on days 14 and 21. The severe polydipsia and polyuria in Li-fed rats were associated with marked decreases in protein abundance of AVP-regulated water channel AQP2 in inner medulla. The abundance of 29-kDa native AQP2 protein molecule in Li-fed rats decreased to about 20% of that in the control group, whereas the abundance of glycosylated forms of AQP2 (35–50 kDa) was decreased to about 15% of the control group on day 21 (Fig. 2). The decrease in the observed changes in water consumption, urine output, urine osmolality, and blood Li levels were not significantly different between day 14 and day 21, the following experiments focused on effects of the Li-added diet for 14 days.

Fig. 1. Polydipsia and polyuria associated with lithium (Li) feeding. Groups of Sprague-Dawley rats (n = 5/group) were fed control or Li-added diet for 21 days. Water intake and urine output were monitored periodically. Body weight (A), water intake (B), urine output (C), and urine osmolality (D) in the 2 groups on days 0, 14, and 21 are shown. Results are means ± SE (n = 5). *Significantly different from corresponding value in control diet group. Mean values of urine output and urine osmolality in Li-fed rats on day 14 and day 21 are not significantly different from each other.

Fig. 2. Decreased protein abundance of aquaporin (AQP)2 associated with Li feeding. Groups of Sprague-Dawley rats (n = 4/group) were fed control or Li-added diet for 21 days and then euthanized. Inner medullas were processed for the determination of AQP2 protein abundance by Western blotting. A: AQP2 protein bands as visualized by Western blotting. B: densitometry values of 29-kDa and 35- to 50-kDa protein bands of AQP2 water channel seen in A. The sharp 29-kDa band corresponds to the native AQP2 protein molecule, whereas the smear from 35 kDa to 50 kDa represents the various glycosylated forms of AQP2 molecule. Results are means ± SE (n = 4). *Significantly different from corresponding value in control diet group.
for the assay of PGE2 metabolite. NDI, we examined whether nucleotide-stimulated PGE2 re-signaling may play a key role in the pathogenesis of Li-induced collecting duct.

To validate our hypothesis that purinergic

collected from rats fed control or Li-added diet (n = 8/group) and processed for the assay of PGE2 metabolite. A: urinary PGE2 metabolite expressed as nanograms per milligram of creatinine. B: urinary excretion of PGE2 metabolite expressed as nanograms per 24 h. Results are means ± SE of triplicate incubations for each condition. *P < 0.01.

Fig. 3. Urinary excretion of prostaglandin (PG)E2 metabolite in rats fed control or Li-added diet for 14 days. Twenty-four-hour urine samples were collected from rats fed control or Li-added diet (n = 8/group) and processed for the assay of PGE2 metabolite. A: urinary PGE2 metabolite expressed as nanograms per milligram of creatinine. B: urinary excretion of PGE2 metabolite expressed as nanograms per 24 h. Results are means ± SE of triplicate incubations for each condition. *P < 0.01.

Urinary excretion of PGE2 metabolite. PGE2 in the urine is not stable. Hence we converted it to a stable metabolite and assayed the metabolite content of the urine samples. Figure 3 shows that the PGE2 metabolite content in Li-fed rats is about twofold higher compared with rats fed the control diet. This is seen for both nanograms per milligram of urinary creatinine and nanograms per 24 h. These data, which support the earlier findings by other investigators (42), indicate that in our model of Li-induced NDI there is also increased production of renal prostaglandins.

Nucleotide-stimulated release of PGE2 by inner medullary collecting duct. To validate our hypothesis that purinergic signaling may play a key role in the pathogenesis of Li-induced NDI, we examined whether nucleotide-stimulated PGE2 release by inner medullary collecting ducts is enhanced in Li-induced NDI. In preliminary experiments we examined the adenosine 5’-O-(3-thiotriphosphate) (ATPγS)-stimulated release of PGE2 by freshly prepared fractions enriched in inner medullary collecting ducts from rats fed control or Li-added diets for 14 or 21 days. We observed that at both time points ATPγS, the universal P2y receptor agonist, caused enhanced PGE2 release by inner medullary collecting ducts of Li-fed rats compared with control diet-fed rats. Furthermore, at both time points, the magnitude of the purinergic-driven enhanced release of PGE2 in Li-fed rats was comparable (data not shown). Hence in the following experiments we studied the effect of feeding the Li-added diet for 14 days only. In addition, we used other P2y receptor agonists, namely UTP and ADP, to understand the nature of the P2y receptor subtype involved. In these experiments, medullary collecting ducts from control diet-fed rats released significantly higher amounts of PGE2 into the incubation medium when stimulated with 50 μM ATPγS or UTP, but not ADP (Fig. 4A). On the other hand, medullary collecting ducts from Li-fed rats released significant amounts of PGE2 into the incubation medium when stimulated by ATPγS, UTP, and ADP (Fig. 4A) compared with the vehicle group. Furthermore, the absolute amounts of PGE2 released by these three nucleotides in Li-fed rats were comparable (Fig. 4A) and were not significantly different from one another. In addition, the amounts of PGE2 released by the medullary collecting ducts of Li-fed rats, both under basal conditions (vehicle) and in response to the three nucleotides, were significantly higher compared with the corresponding releases in the control rats (Fig. 4B). Thus these findings suggest that enhanced purinergic-driven PGE2 release by inner medullary collecting ducts may be an early phenomenon in Li-induced NDI, which runs in parallel to the development of polydipsia, polyuria, and rise in blood Li levels. In addition, since ADP also elicited enhanced PGE2 release by inner medullary collecting ducts of Li-fed rats, it is possible that P2y receptor subtype(s) other than P2y2 may also be a key player in Li-induced NDI.

Fig. 4. Nucleotide-stimulated PGE2 release by inner medullary collecting duct preparations from rats fed control or Li-added diet for 14 days. Fractions enriched in medullary collecting duct were freshly prepared by pooling inner medullas (n = 5 rats/group). Aliquots of fractions were warmed up to 37°C before challenging with each nucleotide at 50 μM for 20 min. Reactions were stopped by adding chilled buffer. PGE2 released into the medium was assayed and normalized to the protein contents of the respective incubations. Results are means ± SE of triplicate incubations for each condition. A: absolute amounts of PGE2 released as nanograms per milligram of protein under each condition. B: PGE2 released by the medullary collecting ducts of Li-fed rats as % over corresponding PGE2 release by control diet-fed rats. Statistical analysis by ANOVA: A, control diet group; *mean values in adenosine 5’-O-(3-thiotriphosphate) (ATPγS) and UTP incubations significantly different from mean value in corresponding vehicle incubations (P < 0.01). A, Li diet group; *mean values in ATPγS, ADP, and UTP incubations significantly different from mean values in corresponding vehicle incubations (P < 0.01).

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Expression of P2y receptor subtypes in the inner medulla.

The qualitative and quantitative differences observed between the inner medullary collecting ducts from control and Li diet-fed rats after nucleotide-induced PGE2 release suggested the involvement of more than one subtype of P2y receptor in Li-fed rats (Table 2). Hence we examined the potential changes in receptor expression by real-time RT-PCR with RNA extracted from the inner medullas. We choose to determine the relative expression of P2y1, P2y2, P2y4, and P2y6 receptors, because these four receptors are coupled to the phosphoinositide signaling pathway and thus are relevant for this study on NDI (Table 2). The results show that in rats fed control diet P2y2 is the predominant P2y receptor subtype expressed in the inner medulla, followed by P2y6 (Fig. 5A). However, after Li diet for 14 days, P2y2 receptor expression was decreased to ~57%, associated with a 3.4-fold (340%) increase in the expression of P2y4 receptor relative to the rats on control diet (Fig. 5B). However, in terms of absolute quantity, the P2y2 receptor remained the predominant P2y receptor subtype even in Li-fed rats. The relative expression of P2y1 and P2y6 receptors did not change after Li feeding. Sequencing of PCR products established the specificity of amplifications for P2y receptors by providing a 98–99% match to the target sequence. Thus these data suggest that both P2y2 and P2y4 receptors may be involved in the enhanced production of PGE2 by inner medullary collecting ducts in Li-induced NDI.

Receptor localization in the inner medulla. Since RT-PCR experiments showed a large increase in the relative expression of P2y4 receptor in the inner medullas of Li-fed rats, we performed confocal laser immunofluorescence microscopy to examine the cellular localization of this receptor protein in the inner medulla. Comparisons between P2y2 and P2y4 were also made. As shown in Fig. 6, the distribution of P2y4 receptor protein is similar to that of P2y2 receptor, i.e., predominantly in collecting ducts, in rats fed control or Li-added diets. In addition, differences in the subcellular distribution of both P2y2 and P2y4 receptors in control and Li-fed rats were observed. However, these aspects are not probed further in this communication. Thus the findings from the confocal immunofluorescence microscopy, when taken in conjunction with data from the nucleotide-stimulated PGE2 release by inner medullary collecting ducts and the quantitative RT-PCR experiments on inner medulla, suggest the potential involvement of both P2y2 and P2y4 receptors in the enhanced purinergic signaling in Li-induced NDI.

DISCUSSION

Although increased production of renal PGE2 has been implicated in the polyuria of Li-induced NDI, the driving force for this is not known. Using a classical model of Li-induced NDI, we documented 1) markedly enhanced nucleotide-driven PGE2 release by the medullary collecting duct; 2) potential involvement of P2y2 and/or P2y4 receptors in the enhanced PGE2 release by medullary collecting duct; 3) marked increase in the expression of P2y4 receptor mRNA in the inner medulla; and 4) predominant localization of P2y2 and P2y4 receptor proteins in the medullary collecting duct. Together, these data suggest that purinergic signaling may play a previously unidentified potential role in the genesis of polyuria of Li-induced NDI by driving the increased production of PGE2 by medullary collecting ducts.

Efficient transport of water, sodium, and urea by the nephron, which is controlled by AVP, is crucial for the urinary concentration mechanism (15). The role of purinergic signaling in opposing the action of AVP on the renal transport of water and solutes is increasingly being recognized (8, 32, 46, 47, 52, 55). In a series of studies in rat models we established the potential role of P2y2 receptor in antagonizing the action of AVP on medullary collecting duct (26–28, 49, 50, 54). Recently, using P2y2 receptor gene knockout mice, we documented (57) that purinergic signaling may play a potential role in the water transport by medullary collecting ducts.

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**Table 2. Agonist potency and signal transduction mechanisms of P2y1, P2y2, P2y4, and P2y6 receptors**

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Amino Acids</th>
<th>Agonist Potency</th>
<th>Signal Transduction Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2y1</td>
<td>362</td>
<td>2-MeS-ATP ≥ ATP &gt;&gt; ADP</td>
<td>Gq/G11; PLC-β†</td>
</tr>
<tr>
<td>P2y2</td>
<td>373</td>
<td>UTP ≈ ATP &gt; ATP[S] &gt;&gt; 2-MeS-ATP</td>
<td>Gq/G11; (Gi/G0?); PLC-β†</td>
</tr>
<tr>
<td>P2y4</td>
<td>352</td>
<td>UDP = UTP &gt; ATP = ADP</td>
<td>Gq/G11; (G0?); PLC-β†</td>
</tr>
<tr>
<td>P2y6</td>
<td>379</td>
<td>UDP &gt; UTP &gt; ADP &gt;2-MeS-ATP &gt;&gt; &gt; ATP</td>
<td>Gq/G11 and G2, PLC-β†</td>
</tr>
</tbody>
</table>

2-MeS-ATP, 2-methylthio-ATP; ATP[S], adenosine 5’-O-(3-thiotriphosphate). Adapted with permission from Schwiebert and Kishore (47), Vallon (52), Burnstock (9), and Abbracchio et al. (1).
overarching role in balancing the effect of AVP on the urinary concentration mechanism.

Previously we demonstrated (54) that inner medullary collecting ducts of normal rats, when stimulated by ATP/S or UTP, produce and release PGE₂, which is consistent with the presence of P₂Y₂ receptor. Interestingly, however, in the present study when rats were fed Li-added diet for 14 days, the nucleotide-stimulated PGE₂ release revealed both qualitative and quantitative differences compared with the PGE₂ release by medullary collecting duct of control diet-fed rats. Qualitatively, the medullary collecting duct from Li-fed rats responded to ADP, in addition to ATP/S and UTP (Fig. 4B), suggesting the potential involvement of P₂Y₆ and/or P₂Y₁ receptor subtypes as well, in addition to P₂Y₂ receptor (Table 2).

Fig. 6. Confocal immunofluorescence localization of P₂Y₂ and P₂Y₄ receptor proteins in the inner medullas of rats fed control or Li-added diets for 14 days. Kidney sections were processed for immunofluorescence microscopy with specific rabbit polyclonal antibodies to P₂Y₂ or P₂Y₄ receptors in conjunction with Cy5-conjugated donkey rabbit IgG. Cy5 was detected with a red HeNe laser at 633 nm. A and C: representative profiles for P₂Y₂ receptor in control and Li diet-fed rats, respectively. B and D: representative profiles for P₂Y₄ receptor in control and Li diet-fed rats, respectively. Sections used for generating A and B were obtained from the same block of paraffin-embedded kidney from a rat fed control diet. Similarly, sections used for generating C and D were obtained from the same block of paraffin-embedded kidney from a rat fed Li-added diet. Arrows indicate red fluorescence of Cy5 in medullary collecting duct. Images only depict localization of P₂Y₂ and P₂Y₄ receptor proteins in the inner medullary structures, and the intensity of the fluorescence does not correspond to the levels of their protein abundance.

Fig. 7. The cascade of events in the biosynthesis of PGE₂ and the potential utility of purinergic antagonists to block the increased production of PGE₂ in Li-induced nephrogenic diabetes insipidus. The availability of free arachidonic acid is often the rate-limiting step in the synthesis of PGE₂ by cyclooxygenases (COX-1 or COX-2). The release of arachidonic acid from membrane phospholipids is critically dependent on the activity of phospholipases. The activation of phospholipases is in turn dependent on signal transduction through G protein-coupled receptors that act through phosphoinositide signaling pathway, such as the P₂Y₆ purinergic receptors. Hence, blocking the activity of P₂Y₆ receptors by the use of specific receptor antagonists should effectively decrease the release of free arachidonic acid for the synthesis of PGE₂ by COX-1 or COX-2, and thus ameliorate Li-induced polyuria.
Quantitatively, the amounts of PGE$_2$ released by the medullary collecting duct of Li-fed rats in response to the three nucleotides tested were significantly higher than the corresponding amounts in control diet-fed rats (Fig. 4B). Together these two findings indicate that Li feeding sensitizes the purinergic signaling in the medullary collecting ducts to extracellular nucleotides. In light of these ex vivo data generated by the use of freshly prepared fractions enriched in inner medullary collecting ducts, it is reasonable to deduce that the twofold increase in urinary PGE$_2$ metabolite seen in Li-induced NDI may be due to increased production of PGE$_2$ by medullary collecting ducts in vivo.

The observed qualitative shift in the purinergic signaling in medullary collecting duct following Li feeding is associated with a marked 3.4-fold increase in the mRNA expression of P$_{2y4}$ receptor, suggesting the potential involvement of this receptor in Li-induced NDI (Fig. 5B). However, the mRNA expression of P$_{2y6}$ receptor, the other potential candidate based on nucleotide-stimulated release of PGE$_2$, was not changed by Li feeding. Despite the observed significant decrease in expression, P$_{2y2}$ receptor still remained the predominant P$_2y$ receptor (Fig. 5A). Furthermore, preliminary data we obtained showed that genetic deletion of P$_{2y2}$ receptor in mice results in marked resistance (~50%) to Li-induced polyuria, indicating the potential involvement of P$_{2y2}$ receptor in the genesis of NDI. In addition, it is well known that the activity of G protein-coupled receptors, such as the P$_{2y}$ receptors, is regulated by a variety of receptor-associated factors independent of receptor expression itself (10, 13, 20). Hence at this stage we cannot rule out the lack of involvement of P$_{2y2}$ receptor in Li-induced NDI based on its mRNA expression alone.

After documenting the potential involvement of P$_{2y4}$ receptor by functional analysis and mRNA expression, we then turned to examine the expression and localization of P$_{2y4}$ receptor protein in the inner medulla by confocal laser immunofluorescence microscopy and compared them with the expression and localization of P$_{2y2}$ receptor. We observed that, similar to P$_{2y2}$ receptor, P$_{2y4}$ receptor protein is expressed in the medullary collecting duct. However, the subcellular distribution of these two proteins apparently differed in control versus Li-fed rats. Pending more detailed studies, in the present work we did not pursue these aspects further.

Less is known about the role of P$_{2y4}$ receptor in the collecting duct biology compared with advances in P$_{2y2}$ receptor biology. Recently Wildman and associates (55), using an approach of whole cell patch clamp of principal cells of split-open collecting ducts from sodium-restricted rats, found that activation of P$_{2y2}$ and/or P$_{2y4}$ receptors inhibited the activity of epithelial sodium channel (ENaC). In parallel, they found that P$_{2y4}$ mRNA levels were insignificant in the microdissected sodium-repleted rats, whereas there was a marked abundance of P$_{2y4}$ receptor mRNA in sodium-restricted rats (55). Hence it is possible that, at least under pathophysiological conditions, the P$_{2y2}$ and P$_{2y4}$ receptors may function synergistically.

Increased expression of cyclooxygenases (COX-1 and COX-2) in Li-induced NDI has been implicated in the increased production of PGE$_2$ (30, 42). However, the availability of free arachidonic acid, the substrate, itself is considered by many investigators as the initial and often the rate-limiting step in the production of PGE$_2$ (4, 22, 37). In this context, our study demonstrates that enhanced purinergic signaling in the medullary collecting duct following Li feeding may hold the key for the increased production of free arachidonic acid from membrane phospholipids via phospholipases as depicted in Fig. 7. The increased expression of COX enzymes in Li-induced NDI may only partially facilitate the rapid formation of PGE$_2$ from the increased availability of free arachidonic acid due to enhanced purinergic signaling.

Finally, the long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs), such as indomethacin, and COX-2-specific inhibitors (celecoxib or rofecoxib), which suppress PGE$_2$ production by directly inhibiting cyclooxygenases, is known to cause permanent renal toxicity and damage and/or elevated serum Li to toxic levels (6, 16, 39, 41). Hence replacement of the current side effect-prone drugs with new drugs based on improved understanding of the molecular pathophysiology of Li-induced NDI should result in improved efficacy and fewer side effects in the clinic. This work represents the first step in that direction. The potential role of purinergic signaling in the enhanced production and release of PGE$_2$ by the medullary collecting duct in Li-induced NDI uncovered by us opens the possibility of targeting purinergic signaling by specific receptor antagonists to decrease the PGE$_2$ production in Li-induced NDI as depicted in Fig. 7.

ACKNOWLEDGMENTS

The authors thank Kenneth E. Hill for his help in confocal microscopy and Rajia Sun and Andrew Hemmert for technical assistance in the initial stages.

Parts of this work were presented at Experimental Biology 2005 (April 2005, San Diego, CA) (51), the Purines 2008 Meeting (June-July 2008, Copenhagen, Denmark) (25), and the 41st Annual Meeting of the American Society of Nephrology (November 2008, Philadelphia, PA) (24) and appeared as printed abstracts in the proceedings of those meetings.

GRANTS

This work was supported by a Department of Veterans Affairs Merit Review Project (to B. K. Kishore), National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-61183 (to B. K. Kishore), the National Kidney Foundation of Utah and Idaho (to B. K. Kishore), and the resources and facilities at the Department of Veterans Affairs Salt Lake City Health Care System.

DISCLOSURE

The proprietary information presented here is protected by an international patent under the Patent Cooperation Treaty (PCT/US2005/038231) and was published by the World Intellectual Property Organization (WO2006/066679).

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