Published in final edited form as:

Acta Physiol (Oxf). 2015 June ; 214(2): 176–188. doi:10.1111/apha.12507.

Targeting Renal Purinergic Signalling for the Treatment of Lithium-induced Nephrogenic Diabetes Insipidus

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Abstract

Lithium still retains its critical position in the treatment of bipolar disorder by virtue of its ability to prevent suicidal tendencies. However, chronic use of lithium is often limited by the development nephrogenic diabetes insipidus (NDI), a debilitating condition. Lithium-induced NDI is due to resistance of the kidney to arginine vasopressin (AVP), leading to polyuria, natriuresis

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Conflict of Interest

We do not have any conflict of interest to declare, except for the fact that we patented our invention.

Disclosures

This review is based on symposium presentations made by B. K. Kishore at the Acta Physiologica Conference on *Renal Purinergic Signalling in Health and Disease*, Uppsala Universitet and the Karolinska Institutet, Uppsala, Sweden (June 13–14, 2014), and the satellite of the Queenstown Molecular Biology Meetings with *The Kidney in Health and Disease Research Theme*, University of Otago, New Zealand (August 28–29, 2014). The use of selective antagonists of P2Y₂ or P2Y₁₂ or related receptors for the treatment of nephrogenic diabetes insipidus is proprietary to the US Department of Veterans Affairs and the University of Utah Research Foundation, and has been protected by the pending or issued patents PCT/US2005/03231 and PCT/US2012/052819.

and kaliuresis. Purinergic signalling mediated by extracellular nucleotides (ATP/UTP), acting via P2Y receptors, opposes the action of AVP on renal collecting duct (CD) by decreasing the cellular cAMP and thus AQP2 protein levels. Taking a cue from this phenomenon, we discovered the potential involvement of ATP/UTP-activated P2Y₂ receptor in lithium-induced NDI in rats, and showed that P2Y₂ receptor knockout mice are significantly resistant to Li-induced polyuria, natriuresis and kaliuresis. Extension of these studies revealed that ADP-activated P2Y₁₂ receptor is expressed in the kidney, and its irreversible blockade by the administration of clopidogrel bisulfate (Plavix®) ameliorates Li-induced NDI in rodents. Parallel in vitro studies showed that P2Y₁₂ receptor blockade by the reversible antagonist PSB-0739 sensitizes CD to the action of AVP. Thus, our studies unraveled the potential beneficial effects of targeting P2Y₂ or P2Y₁₂ receptors to counter AVP resistance in lithium-induced NDI. If established in further studies, our findings may pave the way for the development of better and safer methods for the treatment of NDI by bringing a paradigm shift in the approach from the current therapies that predominantly counter the anti-AVP effects to those that enhance the sensitivity of the kidney to AVP action.

Keywords

Antagonists; purinergic signalling; P2Y₂ receptor; P2Y₁₂ receptor; lithium; collecting duct; arginine vasopressin; diabetes insipidus; water channels; sodium transporters; prostaglandins

A. Prologue

In 1937, while commenting on the complexity of water and salt handling by the mammalian kidney, Homer W. Smith, the celebrated renal physiologist said: “*The history of renal physiology has erred, more often than not, by attempts at oversimplification. The problems of water and salt excretion appear to be extremely complex, and especially liable to this danger.*” Ironically, the same complexity of water and salt excretion by the kidney may also hold the clues to understand disorders of salt and water excretion, and thus helps us to find novel and safer therapies for those disorders, as illustrated in this review. This review is a comprehensive presentation of our focused efforts over the past 15 years to understand the role of purinergic signalling in the molecular physiology of water and solute handling by the kidney, and how it steered us to uncover the potential benefits of targeting renal purinergic signalling for the treatment of lithium-induced nephrogenic diabetes insipidus (NDI), the most common cause of acquired NDI. This review deals with several aspects of renal physiology and pathophysiology in relation to water handling. For those aspects that are well known to most readers, we did not elaborate the details and cited recent reviews. For those aspects that are not within the purview of most readers, we have given details in addition to citing relevant literature.

B. Arginine Vasopressin (AVP) and Nephrogenic Diabetes Insipidus (NDI)

It has been known for a long time that the neurohypophyseal hormone, arginine vasopressin (AVP), commonly known as pitressin or anti-diuretic hormone (ADH), plays a pivotal role in the concentration of urine by the mammalian kidney (Boone & Deen, 2008; Sands & Layton, 2009; Stockand, 2010; Wilson *et al*, 2013). However, major breakthroughs in our understanding of the action of AVP on the kidney occurred starting from the 1990s, thanks

to the cloning of vasopressin V₂ receptor, aquaporin (AQP) water channels, bumetanide-sensitive Na-K-2Cl cotransporter-2 (NKCC2) and the urea transporter (UT) isoforms. AVP, acting through its V₂ receptor and the associated cAMP signalling pathway in the kidney, increases the activity and/or expression of all these transporters/channels, and thus augments urinary concentrating ability of the kidney (Juul *et al*, 2014; Ares *et al*, 2011; Klein *et al*, 2011). Furthermore, the seminal work performed in early 1990s in the laboratory of Dr. Mark Knepper at the NHLBI, NIH, demonstrated reversible translocation of AQP2 protein between the subapical vesicles to apical plasma membrane and vice versa in response to stimulation with or withdrawal of AVP in microperfused rat inner medullary collecting ducts (Nielsen *et al*, 1995). The subsequent studies from several laboratories have been deciphering the intricate mechanisms of AVP-V₂ receptor-cAMP-AQP2 axis in the regulation of water transport in health and disorders of water balance (Radin *et al*, 2012; Wilson *et al*, 2013; Fenton *et al*, 2013). Thus, the last two decades have seen an exponential increase in our fund of knowledge of renal transport physiology in general, and the urinary concentrating mechanism in particular.

Despite these advancements, one particular aspect of AVP action that continues to raise the eyebrows of renal physiologists or pathophysiologists is the so called “vasopressin resistant states”, whereby the kidney becomes resistant to the action of AVP (Hays & Leaf, 1961; Ball, 2007). This phenomenon has several clinical correlates, which are commonly known by the generic term “acquired NDI”, as opposed to the “inherited NDI”, in which the defect is in the gene coding for V₂ receptor or AQP2 water channel (Oksche & Rosenthal, 1998; Sands & Bichet, 2006). Clinically acquired NDI is a socially inconvenient, debilitating and morbid condition, characterized by polydipsia, polyuria, natriuresis and kaliuresis among others. There are two categories of acquired NDI, depending on how narrowly or broadly one applies the definition of acquired NDI. In the narrow definition, the water permeability of the collecting duct is not increased by AVP. This category comprises the lithium-induced NDI, and NDI due to hypokalemia, hypercalcemia, and post-obstructive uropathy, as well as therapeutic usage of cisplatin, demeclocycline, and amphotericin B. The broader definition of NDI refers to defective medullary countercurrent function, and it involves a variety of diseases, such as renal failure (acute or chronic), amyloidosis, sarcoidosis, Sjögren syndrome, protein malnutrition, cystinosis, sickle-cell anemia and trait, and the use of aminoglycosides, and loop diuretics.

C. Purinergic Signalling antagonizes the Action of AVP in the Collecting Duct

While the mechanisms of action of AVP, a systemic hormone, on the regulation of water and sodium are being deciphered, in parallel a variety of autocrine and paracrine agents have been identified and shown to regulate renal water and sodium handling. The most widely studies autocrine/paracrine agents, which fall under the broad “intrinsic mechanisms” in the kidney are prostaglandin E₂ (PGE₂), endothelin, and extracellular nucleotides (ATP/UTP). These agents, acting through their respective G protein-coupled receptors, and the associated signalling pathways, often decrease the cellular cAMP levels, and thus counter the action of AVP on the kidney, especially in the collecting duct (Olesen & Fenton, 2013; Kohan, 2011;

Kishore *et al*, 2009; Rieg & Vallon, 2009; Vallon & Rieg, 2011; Breyer *et al*, 2013; Shirley *et al*, 2013). Today, the field of autocrine or paracrine regulation of renal transport of water and sodium is one of the hot topics in renal research, with several groups all over the world studying various aspects and implications of these agents. The general consensus is that the intrinsic regulation of renal water and sodium transport by the autocrine/paracrine agents may hold the key for the so called vasopressin resistant states, which appears to be true based on several published studies.

Purinergic signalling, mediated by extracellular nucleotides, such as ATP/ADP/UTP, is a relatively new area of research within the intrinsic mechanisms in the kidney (Schwiebert & Kishore, 2001). The most widely studied purinergic receptor in the kidney is ATP/UTP activated P2Y₂ receptor, which is expressed in the collecting duct and several other structures of the kidney (Kishore *et al*, 2000). Studies from our laboratories and others have shown that signalling mediated through P2Y₂ receptor antagonizes the action of AVP on the collecting duct, and thus decreases water transport (Kishore *et al*, 2009; Rieg *et al*, 2007; Zhang *et al*, 2008; Rieg & Vallon, 2009; Prætorius and Leipziger, 2010; Vallon & Rieg, 2011; Shirley *et al*, 2013). Figure 1 illustrates the potential intracellular mechanisms involved in the antagonistic effect of P2Y₂ receptor on the AVP-mediated water flow in the medullary collecting duct. Briefly, P2Y₂ receptor is a G_i/G_q-coupled extracellular nucleotide receptor with an agonist potency order of UTP > ATP > ATP γ S $\gg$ 2-MeS-ATP. Agonist activation of P2Y₂ receptor on the basal aspect of the collecting duct results in enhanced activity of phosphoinoside signalling pathway, and the associated generation of inositol 3-phosphate (IP₃) and diacylglycerol (DAG). DAG is known to activate protein kinase C (PKC), which, acting through inhibitory G protein (G_i), uncouples signal transduction from V₂ receptor to adenylyl cyclase (AC) even in the presence of AVP. Accordingly, in isolated microperfused medullary collecting ducts of rat it has been shown by Kishore, Chou and Knepper that ATP applied on the basal aspect significantly reduced AVP-induced osmotic water permeability, and this was blocked by PKC inhibitor calphostin C (Kishore *et al*, 1995). PKC also potentially activates cytosolic phospholipase A₂ (cPLA₂), which releases free arachidonic acid, the rate-limiting substrate for the synthesis of PGE₂ by cyclooxygenases (COX). Accordingly, in a model of acutely isolated fractions of rat inner medullary collecting ducts we have shown that ATP or UTP enhances the production of PGE₂, which was specifically blocked by COX-1 inhibition (Welch *et al*, 2003). PGE₂ thus produced is transported out of the cells through prostaglandin transporter proteins, and acts on the E-prostanoid receptors, especially the EP₃ subtype, which is abundantly expressed in the medullary collecting ducts. Activation of EP₃ receptor is known to downregulate cellular cAMP levels. Thus, the activation of P2Y₂ receptor has dual antagonizing effects on AVP action – one is direct interference in the activity of AC through DAG-PKC-G_i pathway, and another indirectly by enhancing the production of PGE₂. Apart from these two, there is a third potential pathway for which experimental evidence does not exist beyond the increase in the cytosolic free [Ca²⁺] levels (Ecelbarger *et al*, 1994). Increased cytosolic [Ca²⁺] levels are known to activate phosphodiesterases (PDE) through calcium-calmodulin (CaM) pathway, and thus decrease cellular cAMP levels (Sharma *et al*, 2006). Hence, the effect of P2Y₂ receptor activation on this pathway needs to be investigated.

As shown in the Figure 1, P2Y₂ receptor is also expressed on the apical domain of the medullary collecting duct. But it appears that the apical P2Y₂ receptor is not involved in the regulation of water permeability (Edwards, 2002). On the other hand, the apical P2Y₂ receptor is involved in the regulation of sodium absorption through the epithelial sodium channel (ENaC) (Wildman *et al*, 2008). Elegant patch clamp studies performed on the apical membrane of principal cells in split-open medullary collecting ducts of mice by Dr. James Stockand and associates have shown the regulatory role of apical P2Y₂ receptor on the activity of ENaC (Mironova *et al*, 2015).

Since collecting duct is the only renal segment where the absorption of water and sodium are not coupled and can be independently regulated by AVP or aldosterone, respectively, the expression and function of P2Y₂ receptor on the basal and apical aspects of P2Y₂ receptor bestow it with a unique functional significance. The basal expression opposes the AVP action while the apical expression opposes the aldosterone action. Thus P2Y₂ receptor can independently modulate the functions of both hormones, depending on the availability of the agonists (ATP/UTP) in the extracellular milieu. The mechanisms involved in the regulated release of the nucleotides by collecting duct cells are yet to be deciphered, although the potential nucleotide transporters have been identified (Schwiebert, 2001; Sipos *et al*, 2009; Burnstock & Verkharastsky, 2012; Svenningsen *et al*, 2013).

D. Lithium and Nephrogenic Diabetes Insipidus (NDI)

Introduced into the modern psychiatric medicine in 1949 by John Cade, an Australian psychiatrist, the alkali metal lithium proved to be the most efficacious drug for the treatment of bipolar disorder and related manic illnesses (Cade, 1949; Mitchell, 1999). Bipolar disorder, often a sequel of post-traumatic stress disorder (PTSD) has a prevalence of 2% in the general population and twice that number in war Veterans in the United States. Due to the recent and ongoing wars in Afghanistan and Iraq, there has been a steep rise in the prevalence of PTSD among returning soldiers (Hoge *et al*, 2004; Sundin *et al*, 2010). Despite the introduction of newer drugs for the treatment of bipolar disorder, lithium retained its prime position by virtue of its ability to prevent suicidal tendencies, a common occurrence in bipolar patients (Baldessarini *et al*, 2006; Hirschowitz *et al*, 2010 ; Novoli *et al*, 2010). Currently about 30% of the bipolar patients receive lithium therapy, where it has distinct advantages over the non-lithium drugs. In addition to its proven benefits in bipolar patients, in recent years lithium has emerged as a robust neuroprotective agent for the treatment of acute brain injury (e.g., stroke or ischemia), and chronic neurodegenerative diseases, such as Alzheimer's, Parkinson's, and Huntington's (Manji *et al*, 1999; Rowe & Chuang, 2004; Chuang, 2004; Wada *et al*, 2005; Young *et al*, 2009; Quiroz *et al*, 2010). This is because, by virtue of its ability to promote anti-apoptotic pathways, lithium counters apoptosis of brain cells. At present, lithium is the only known substance that stimulates antiapoptotic pathways in the brain. In view of these beneficial properties of lithium in bipolar disorder, acute brain injury and neurodegeneration, it is destined to stay in the market for a long time.

Despite its therapeutic value, chronic use of lithium is often limited by its adverse effect on the kidney, leading to the development of nephrogenic diabetes insipidus (NDI). About 40%

of lithium-treated patients develop NDI, which manifests as AVP-resistant polyuria. Studies in animal models have revealed that lithium-induced polyuria is due to marked decrease in AVP-regulated AQP2 water channel in the renal collecting duct (Marples *et al*, 1995), the mechanisms of which are not completely understood. Studies have also shown or suggested that the underlying cause can be inhibition of glycogen synthase kinase-3, β -isoform (GSK3 β) or impaired cAMP production or dysregulation of renal prostaglandin production, and others (Sugawara *et al*, 1988; Weinstock & Moses, 1990; Rao *et al*, 2005; Jia *et al*, 2009). In human patients and animal models, chronic administration of lithium has also been shown to induce collecting duct remodeling (increase in proportion of intercalated cells relative to the proportion of principal cells), cell proliferation, and interstitial fibrosis (Hansen *et al*, 1979; Christensen *et al*, 2004; Christensen *et al*, 2006; Walker *et al*, 2013). In human patients, chronic usage of lithium is known to reduce glomerular filtration rate (GFR), leading to chronic kidney disease (CKD) (Presen *et al*, 2003). For a comprehensive presentation of the renal effects of lithium, please see reviews by us (Kishore & Ecelbarger, 2013) and others (Gitlin, 1994; Grünfeld & Rossier, 2009).

Currently used approaches for the treatment of lithium-induced NDI, such as the combined use of a thiazide and amiloride or non-steroidal anti-inflammatory drugs (NSAID; e.g., indomethacin) or selective COX inhibitors, are associated with serious side effects. Thiazides, which reduce GFR (glomerular filtration rate) by activating the tubuloglomerular feedback (TGF) and thus decrease polyuria, should be used with caution as they reduce renal excretion of lithium, resulting in lithium intoxication (Finley *et al*, 1995). Amiloride, a potassium-sparing blocker of the epithelial sodium channel (ENaC), interferes with the uptake of lithium by the collecting duct principal cells, and thus ameliorates polyuria. However, amiloride enhances lithium-induced natriuresis (Bedford *et al*, 2008; Kortenoeven *et al*, 2009). Indomethacin, which suppresses lithium-induced increase in PGE₂ and thus relieves polyuria, often causes headache and dizziness and increased risk of gastrointestinal disorder and bleeding, as well as high blood lithium levels (Murray & Brater, 1993; Phelan *et al*, 2003). Currently, the safety of long-term use of COX-2 selective inhibitors is debated (Conaghan, 2012). It is obvious that these treatment modalities are not suitable or tolerable in elderly and/or high risk patients. Hence, there is a need for better and safer methods of treatment for the lithium-induced NDI.

E. Early Evidence for the Involvement of Purinergic Signalling in NDI

Our early studies in rat models provided significant and intriguing insights into the interactions of purinergic signalling mediated by P2Y₂ receptor with prostanoid and AVP systems in renal collecting ducts under normal conditions, and how these interactions are altered in acquired NDI induced by lithium administration or by post-obstructive uropathy (bilateral ureteral obstruction and release). These series of studies have been comprehensively reviewed in Kishore et al (2009), and schematically illustrated in Figure 2. As illustrated in the scheme, the interaction between the purinergic signalling mediated by P2Y₂ receptor and AVP can be acute (pathway A) or chronic (pathway B). In acute interaction, agonist activation of P2Y₂ receptor down regulates AVP-induced water flow and cAMP production in medullary collecting ducts (Kishore *et al*, 1995). On the other hand, chronically elevated AVP levels (water deprivation or dDAVP infusion) down

regulate the expression and activity of P2Y₂ receptor (Kishore *et al*, 2005; Sun *et al*, 2005a; Sun *et al*, 2005b). The purinergic-mediated prostanoid production in the medullary collecting ducts (pathway C), which is seen in normal conditions (Welch *et al*, 2003), is augmented in acquired NDI (Zhang *et al*, 2009; Zhang *et al*, 2010). The tonic inhibitory effect of PGE₂ on AVP action (pathway D), seen in normal conditions, is augmented in acquired NDI due to enhanced production of PGE₂ (Zhang *et al*, 2009; Zhang *et al*, 2010). Finally, the inhibitory effect of increased AVP levels on purinergic-mediated PGE₂ production (pathway E) seen in normal rats (Sun *et al*, 2005b) is apparently blunted in acquired NDI. These significant interactions among the AVP and purinergic and prostanoid signalling in normal conditions and their alterations in acquired NDI led us to the hypothesis that purinergic signalling is involved in the AVP resistance seen in acquired NDI.

F. Role of P2Y₂ Receptor in Lithium-induced NDI

The availability of mice lacking P2Y₂ receptor (Cressman *et al*, 1999; Homolya *et al*, 1999) allowed us to test our hypothesis that purinergic signalling mediated by P2Y₂ receptor is involved in the development of AVP resistant polyuria in NDI. Accordingly, we found that lithium-induced (2 weeks treatment) polyuria, decrease in urine osmolality and AQP2 protein abundance in the renal medulla were significantly low in mice lacking P2Y₂ receptor as compared to the syngeneic wild type mice (Zhang *et al*, 2012). This protection afforded by genetic deletion of P2Y₂ receptor was not due to decreased intake or absorption of lithium in the gut, as assessed by serum lithium levels, or lesser accumulation of lithium in the inner medulla. Paradoxically, lithium-induced increased urinary excretion of PGE₂ was not affected in the knockout mice (Zhang *et al*, 2012), a finding that contradicted our original hypothesis based on rat models, where we observed that NDI induced by lithium or post-obstructive uropathy were associated with enhanced activity of P2Y₂ receptor, resulting in increased production of renal PGE₂ (Zhang *et al*, 2009 & 2010). To solve this paradox we probed the prostanoid receptor expression and signalling in the medullary collecting duct, since it is not the absolute amount of PGE₂, but the PGE₂ (EP) receptor signalling that matters. We found that prostanoid EP₃ receptor protein abundance in the renal medulla of P2Y₂ receptor knockout mice was markedly low vs. wild type mice, irrespective of whether they were administered lithium or not. The protein abundances of other EP receptors known to be expressed in the collecting duct (EP₁ or EP₄) were not altered. EP₃ receptor activation by PGE₂ decreases the cellular cAMP levels. Hence, a marked decrease in EP₃ receptor protein, without changes in other EP receptors could offset the balance between cAMP and phosphoinositide/Ca²⁺ signalling pathways. Accordingly, ex vivo stimulation of medullary collecting duct with PGE₂ generated significantly more cAMP in lithium-treated knockout mice vs. lithium treated wild type mice. Taken together, these data suggest: (i) genetic deletion of P2Y₂ receptor offers significant resistance to the development of lithium-induced polyuria, and (ii) this resistance is apparently due to altered PGE₂ signalling mediated by a marked decrease in EP₃ receptor protein abundance in the renal medulla, thus attenuating the EP₃-mediated decrease in cAMP levels in the medullary collecting duct (Zhang *et al*, 2012). Thus, it appears in the absence of P2Y₂ receptor, collecting duct alters its response to PGE₂ through regulation of EP₃ receptor. Our findings also suggest that P2Y₂ receptor may be a regulator of EP₃ protein abundance.

Clinically, lithium-induced NDI is also characterized by natriuresis (loss of sodium) and kaliuresis (loss of potassium), in addition to AVP-resistant polyuria (loss of water). The underlying cause(s) for lithium-induced natriuresis and kaliuresis is(are) not understood well, although one would be tempted to rationalize that lithium through its effects on the activity of AVP and/or aldosterone may inhibit sodium and potassium absorption in the kidney, leading to natriuresis and kaliuresis. Since, it has been shown that P2Y₂ receptor opposes the activity of both AVP and aldosterone, we hypothesized that P2Y₂ receptor knockout mice would be less sensitive to lithium-induced natriuresis and/or kaliuresis, due to attenuated downregulation of one or more major sodium or potassium transporter/channel proteins. Accordingly, administration of lithium for 2 weeks resulted in significantly attenuated natriuresis and kaliuresis in P2Y₂ receptor knockout mice vs. wild type mice (Zhang *et al*, 2013). To gain insights into the underlying mechanisms, using semi-quantitative immunoblotting we analyzed the protein abundances of sodium and potassium channels/transporters or exchangers along the nephron and collecting duct. In parallel we assessed the functional status of the transporters/channels by acutely challenging them with nephron segment or collecting duct-specific diuretic agents. Our results suggest that the attenuated natriuretic response to lithium in the P2Y₂ receptor knockout mice was not due to enhanced aldosterone-sensitive distal nephron Na⁺ absorption through ENaC. On the other hand, we could not rule out a role for enhanced Na⁺ reabsorption in AVP-sensitive medullary thick ascending limb (mTAL). It is because, we found that medullary levels of NKCC2 protein were significantly higher in knockout mice, and were not decreased by lithium treatment. Furthermore, P2Y₂ receptor knockout mice had higher medullary tissue osmolality and greater response to furosemide under basal conditions. These results suggest that increased expression and/or activity of the NKCC2 in the P2Y₂ knockout mice might reduce Na⁺ delivery to the distal nephron and collecting duct, and act like a buffer to compensate the loss of Na⁺ in the distal nephron and collecting duct during lithium administration. With regard to kaliuresis, we found a reduction in the secretory, flux-activated BK (potassium) channel protein in the medulla in the knockout mice. But its significance as a contributor for attenuated kaliuresis in the P2Y₂ receptor knockout mice needs to be established. Finally, our findings on the attenuated natriuresis and kaliuresis in P2Y₂ receptor knockout mice have clinical significance and impact. Natriuresis and kaliuresis can activate mechanisms that result in renal lithium retention leading to lithium intoxication, which can become a vicious cycle. In this context, it appears that the observed significant amelioration of lithium-induced polyuria, natriuresis and kaliuresis is unique to the deletion of P2Y₂ receptor. At least two other currently used therapeutic modalities for lithium-induced polyuria, namely, the administration of amiloride or COX-2 inhibition, do not have significant effect on lithium-induced natriuresis and kaliuresis. Furthermore, amiloride treatment markedly enhanced lithium-induced natriuresis, whereas COX-2 inhibition did not have any effect on urinary excretion of Na or K in lithium-treated rats (Bedford *et al*, 2008; Kortenoeven *et al*, 2009).

Finally, treatment of bipolar disorder in human patients requires long-term administration of lithium, for several years. Hence, any proposed new therapy, such as targeting the P2Y₂ receptor should be effective over the time. To address this question, in preliminary studies we administered lithium to P2Y₂ receptor knockout and wild type mice for 5 months

(equivalent to several years in humans) and found that the significant protection afforded by deletion of P2Y₂ receptor against lithium-induced polyuria, natriuresis and kaliuresis is long lasting (Heiney *et al*, 2014).

G. Current Limitations on the Therapeutic Use of P2Y₂ Receptor

Antagonists

Our studies on P2Y₂ receptor knockout mice brought out promising information on the potential utility of targeting this receptor for the treatment of lithium-induced NDI. However, currently there are no FDA-approved or commercially available experimental drugs that can selectively block P2Y₂ receptor in vivo. Although several P2Y₂ antagonists have been described in literature including flavonoids, natural products with an allosteric mechanism of action (Kaulich *et al*, 2003), anthraquinones (Weyler *et al*, 2008; Hillmann *et al*, 2009), and uracil derivatives (Sauer *et al*, 2009), no antagonist with high nanomolar potency and selectivity has been reported. The most useful P2Y₂ antagonist described so far is the thiouracil derivative AR-C118925 obtained by structural modification of the agonist UTP (Kemp *et al*, 2003; Brunschweiler & Müller, 2006). However, until recently AR-C118925 is not commercially available, and as a result, it has not been comprehensively characterized. The development of potent and selective P2Y₂ receptor antagonists with drug-like properties will be indispensable to advance the field.

H. Role of P2Y₁₂ Receptor in Lithium-induced NDI

In view of the current status and limitations in therapeutic applications of P2Y₂ receptor antagonism for the treatment of lithium-induced NDI, we focused our studies on the ADP-activated P2Y₁₂ receptor, that has the potential to oppose AVP-induced water flow in the collecting duct, and for which a time-tested FDA-approved drug is available in the market to block the activity. P2Y₁₂ receptor is a G protein-coupled ADP receptor expressed predominantly in blood platelets, and microglia and astrocytes in the brain. Phylogenetically it is distinct from the P2Y₂ and related receptors, such as P2Y₄ and P2Y₆ (Fig 3). As illustrated in Figure 4, signalling through P2Y₁₂ receptor is mediated through G_i, inhibiting adenylyl cyclase (AC), and activation of class I phosphoinositol 3-kinase (PI3K) (Nguyen *et al*, 2005). P2Y₁₂ receptor signalling is also coupled to G_q, which through phospholipase C increases intracellular Ca²⁺ needed for platelet activation and clot formation (Kalantizi *et al*, 2012). ADP-induced platelet aggregation is initiated by another G protein-coupled receptor, the P2Y₁ receptor, expressed in the platelets, but amplified and sustained by P2Y₁₂ receptor in synergistic manner. As reviewed previously, inhibition of AC activity and activation of phospholipase C are known to decrease the sensitivity of the collecting duct to AVP (Kishore *et al*, 2009). Hence, we hypothesized that if expressed in the kidney, P2Y₁₂ receptor has the potential to play a significant role in the renal handling of water and in pathophysiology of AVP resistant states, such as the acquired NDI.

The availability of an FDA-approved and time tested drug, clopidogrel bisulfate (Plavix®; Bristol-Myers Squibb & Sanofi Aventis), to selectively block P2Y₁₂ receptor in vivo allowed us to test our hypothesis in rodent models. Clopidogrel is an oral thienopyridine class of antiplatelet drug that irreversibly inhibits P2Y₁₂ receptor. It is a pro-drug activated

in the liver by cytochrome P450 enzymes (CYP2C19) generating its active metabolite (Act-Met) which constitutes about 15% of the ingested drug molecule. The Act-Met acts by forming disulfide bridges with the P2Y₁₂ receptor (Kalantizi *et al*, 2012; Zhang *et al*, 2014). Plavix® has been widely used in the clinical practice since 1997 as an anti-clotting agent to prevent cardiovascular or cerebrovascular events (stroke or heart attack) in high-risk patients, and it has been well tolerated with very few side effects.

Since clopidogrel is a pro-drug activated in the liver, it is not suitable for use in cell cultures and in vitro experiments. Hence, for in vitro experiments we used PSB-0739 (1-amino-4[4-phenyl-amino-3-sulfophenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate), a highly potent, selective, reversible non-nucleotide antagonist of P2Y₁₂ receptor that is not toxic to cells (Baqi *et al*, 2009; Hoffmann *et al*, 2009). Unlike clopidogrel, PSB-0739 does not require bioactivation. PSB-0739 was designed, synthesized, purified, and characterized by Prof. Christa E. Müller and coworkers at the University of Bonn, Bonn, Germany (Baqi *et al*, 2010).

Using real-time RT-PCR and gene specific primers, we detected the mRNA expression of P2Y₁₂ receptor in all the three regions – cortex, outer and inner medullas – of rat kidney. To immunolocalize the receptor protein in the kidney, we designed, generated and characterized a C-terminal peptide-derived rabbit polyclonal antibody specific for P2Y₁₂ receptor (Zhang *et al*, 2015). Western blots showed P2Y₁₂ receptor protein in the cortex and outer and inner medullas of rat kidneys. Immunoperoxidase and immunofluorescence (IF) revealed labeling for P2Y₁₂ receptor on the brush border of the proximal tubules in the cortex, and on the apical domain of collecting ducts in the cortex and medulla, as well as in arterioles. Confocal immunofluorescence confirmed P2Y₁₂ receptor protein in AQP2 positive cells in the cortex and medulla, with an apparent localization of both proteins on the apical membrane of the collecting duct cells. Similar observations were made in immunofluorescence when we used a P2Y₁₂ receptor antibody from a reliable commercial source (Alomone Labs). Thus, our observations revealed the expression of P2Y₁₂ receptor in the kidney, especially in the collecting duct, the seat of AVP-regulated water transport.

When administered to rats in drinking water, clopidogrel bisulfate (20 mg/kg bw/day, for 2 weeks) significantly ameliorated polydipsia and polyuria, and reversed the increase in electrolyte-free water excretion induced by lithium. These parameters in water balance were matched with a significant improvement in lithium-induced decrease in AQP2 protein in the inner medulla. However, unlike P2Y₂ receptor deletion, clopidogrel administration had no effect on lithium-induced sodium excretion. Clopidogrel treatment augmented the increased urinary AVP excretion induced by lithium. Finally, clopidogrel did not decrease lithium levels in the blood or in the inner medulla (Zhang *et al*, 2015). Thus, our studies indicate that pharmacological blockade of P2Y₁₂ receptor potentially ameliorates lithium-induced polyuria.

Interestingly, we also observed that administration of clopidogrel alone increased the urinary concentrating ability associated with increased AQP2 protein abundance in the kidney, and increased urinary excretion of AVP, a surrogate for circulating levels of AVP. Based on these findings one may surmise that the protection afforded by clopidogrel against lithium-

induced polyuria in part is related to its ability to induce AVP secretion. But that may not be the case, considering the fact that by definition acquired NDI is resistance of the kidney to the action of AVP. Hence, we examined the possible scenarios which revealed the following interesting phenomena. First, we observed that P2Y₁₂ receptor mRNA is expressed in rat hypothalamus, and exposure of cultured rat hypothalamic cells to PSB-0739, a selective blocker of P2Y₁₂ receptor, resulted in significant increase in the AVP mRNA expression (Zhang *et al*, 2015). This finding may explain the increased urinary excretion of AVP in clopidogrel treated rats. Second, in primary cultures of rat inner medullary collecting duct (IMCD) cells, where we clamped the concentration of dDAVP (desmopressin; a V₂ receptor selective analogue of AVP), PSB-0739 potentiated the effect of dDAVP on the expression of AQP2 and AQP3, and on cAMP production. Interestingly, in the absence of dDAVP, PSB-0739 had no effect on the expression of AQP2 or AQP3 in the cultured IMCD cells. In parallel we observed that clopidogrel administration has no effect on the urinary concentration in Brattleboro mutant rats that lack AVP. Finally, the observed effects of clopidogrel were not mediated through P2Y₂ receptor as an off-target effect, because when administered to P2Y₂ receptor knockout and syngeneic wild type mice, clopidogrel caused comparable increase in urinary concentration (Zhang *et al*, 2015).

Taken together, the above observations strongly suggest that blockade of P2Y₁₂ receptor potentiates the effect of AVP/dDAVP on the kidney/collecting duct by removing the tonic inhibition. But, in the absence of AVP, P2Y₁₂ receptor blocked per se is not sufficient to alter the urinary concentrating ability as we observed in Brattleboro mutant rats. Since lithium-induced NDI is due to AVP resistance of the kidney, the pharmacological blockade of P2Y₁₂ receptor might have relieved the inhibition, and thus sensitized the collecting duct to the action of the increased AVP levels in rats treated with a combination of lithium and clopidogrel. If established in further studies, this potential mechanism may offer a novel approach for the treatment of lithium-induced NDI by relieving the AVP resistance.

I. Summary and Future Directions

Our focused studies on the physiology and pathophysiology of purinergic signalling mediated by P2Y₂ and P2Y₁₂ receptors in renal handling of water revealed the potential involvement of these two receptors in vasopressin-resistant states, such as lithium-induced NDI. It is also clear that genetic suppression or pharmacological blockade of the activity of these receptors increased the urinary concentrating ability of the kidney, thus suggesting that under basal conditions these receptors might be exerting a tonic inhibition on the action of AVP. What is more interesting is the fact that suppression or blockade of these receptors ameliorated the lithium-induced polyuria, by apparently sensitizing the kidney and/or the collecting duct to the action of AVP. If established in further studies, this phenomenon shifts the current focus of research and therapies for lithium-induced NDI from predominantly the ones that counter anti-AVP effects of lithium to the ones that enhance the sensitivity of the kidney to AVP action, i.e., relieves the AVP resistant state in the NDI.

Our future studies are focused on: (i) development of selective, potent and safe P2Y₂ receptor antagonists for in vivo use for the treatment of lithium-induced NDI; (ii) evaluation of long-term protection afforded by P2Y₁₂ receptor blockade on lithium-induced NDI; (iii)

experimenting on the potential utility of concurrent blockade of both P2Y₂ and P2Y₁₂ receptors using receptor selective antagonists for the treatment of lithium-induced NDI; and (iv) probing the potential utility of purinergic antagonism (P2Y₂ and/or P2Y₁₂) in other forms of acquired NDI, such as the ones induced by hypokalemia and hypercalcemia.

Acknowledgments

Thanks are due to Drs. Mark A. Knepper, Simon C. Robson and Jeff M. Sands for critical reading of the manuscript and for valuable suggestions. The authors thank Dr. Beverly Koller of the University of North Carolina at Chapel Hill, Chapel Hill, NC for generously providing the breeders of P2Y₂ receptor knockout mice. Authors' work cited in this review has been supported by grants from the US Department of Veterans Affairs, the National Institutes of Health, the National Kidney Foundation of Utah and Idaho, Catalyst Grant Program of the University of Utah, and the resources and facilities at the VA Salt Lake City Health Care System. B. K. Kishore, N. G. Carlson and D. E. Kohan have been supported by VA Merit Review Program. C. M. Ecelbarger has been supported by a Marriott Cardiovascular Fellowship and an Established Investigator Award from the American Heart Association. Additional support includes National Institute of Diabetes, and Digestive and Kidney Diseases Grant DK-082507 (to C. M. Ecelbarger), DK-64324 & DK-10094 (to J. Peti-Peterdi), and DK-097007 (to D. E. Kohan).

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Figure 1.

Schematic representation of the major G protein-coupled receptors and the channels involved in regulated reabsorption of water and sodium in the principal cell of the collecting duct. The scheme also depicts the major points of interaction between mutually opposing the cyclic AMP and phosphoinositide signaling pathways through which these receptors act. For details refer to the text. (reproduced with permission, from Kishore et al, *Purinergic Signalling* 5:491–499, 2009). AVP – arginine vasopressin; ET – endothelin; PGE₂ – prostaglandin E₂; V2-R – vasopressin V₂ receptor; ET-R – endothelin receptor; EP₃-R – prostanoid receptor type 3; AC – adenylyl cyclase; PLC – phospholipase C; G_s – stimulatory G protein; G_i – inhibitory G protein; cAMP – cyclic AMP; PKA – protein kinase A; PKC – protein kinase C; PDE – phosphodiesterases; IP₃ – inositol triphosphate; ER – endoplasmic reticulum; CaM – calcium calmodulin; DAG – diacyl glycerol; cPLA₂ – cytosolic phospholipase A₂; AQP2, AQP3 and AQP4 – aquaporin water channel isoforms 2, 3 and 4; ENaC – epithelial sodium channel, α, β, γ subunits; Aldo – aldosterone; PIP₂ – phosphatidy-linositol 4,5-bisphosphate; PI3-K – phosphoinositide 3-kinase

Figure 2. Interactions among purinergic signaling (AVP), and AVP and prostanoid (PGE2) systems in medullary collecting duct principal cell under normal conditions (left), and how they are deranged in acquired NDI (right). (–) and (+) signs denote inhibition and stimulation, respectively. X marks indicate blocked pathways. Thicker arrows indicate accentuation of pathways. Thinner arrows indicate attenuation of pathways. Black arrow stands for the pathway already known in the literature. Red arrows stand for the pathways deciphered and reported by us. For the details of the pathways marked by A, B, C, D and E, please refer to the relevant section of the text. (reproduced with permission, from Kishore *et al*, 2009, *Purinergic Signalling* 5:491–499).

Figure 3.

Phylogenetic relation of human P2Y receptors. The P2Y₁₂-like receptors cluster into a group (framed) that is distinct from other nucleotide receptors such as P2Y₁, P2Y₂, P2Y₄ & P2Y₆. (reproduced with permission, from Schöneberg *et al*, 2007, *Purinergic Signalling* 3:255–268).

Figure 4.

P2Y₁₂, P2Y₁ and P2X₁ receptor signalling in platelets. For details, please refer to the text. (reproduced with permission from Nguyen *et al*, 2005, *J Am Coll Cardiol* 45:1157–1164)