P2Y2 receptor mRNA and protein expression is altered in inner medullas of hydrated and dehydrated rats: relevance to AVP-independent regulation of IMCD function

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Departments of 1Medicine and 2Physiology, and 4Division of Pediatric Nephrology, University of Utah Health Sciences Center, Salt Lake City; 3Nephrology Research, Veterans Affairs Salt Lake City Health Care System, Salt Lake City, Utah; and 5Department of Biology, University of Dayton, Dayton, Ohio

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Kishore, Bellamkonda K., Carissa M. Krane, R. Lance Miller, Huihui Shi, Ping Zhang, Andrew Hemmert, Rujia Sun, and Raoul D. Nelson. P2Y2 receptor mRNA and protein expression is altered in inner medullas of hydrated and dehydrated rats: relevance to AVP-independent regulation of IMCD function. Am J Physiol Renal Physiol 288: F1164–F1172, 2005. First published February 1, 2005; doi:10.1152/ajprenal.00199.2004.—Arginine vasopressin (AVP), acting through a cAMP second messenger system, regulates osmotic water permeability (Pf) of the collecting duct. In the collecting duct, the activities of CAMP and phosphonositides (PI) are mutually inhibitory. The P2Y2 receptor (P2Y2-R) is a G protein-coupled extracellular nucleotide receptor associated with PI signaling pathway. Previously, we showed that P2Y2-R is expressed in inner medullary collecting duct (IMCD) of rat, and its agonist (ATP/UTP) activation decreased AVP-induced Pf and resulted in enhanced production of prostaglandin E2 (PGE2). Hydrated and dehydrated states are associated with alterations in the circulating levels of AVP, expression, and/or subcellular distribution of AVP-regulated aquaporin-2 water channel in IMCD and thus Pf of IMCD. We hypothesized that altered expression and/or signaling via P2Y2-R may also modulate IMCD function in these conditions. Sprague-Dawley rats were subjected to dehydration by water deprivation (48 h) or hydration (48 or 96 h) by providing sucrose water. Hydration or dehydration resulted in marked alterations in mRNA expression (Northern blot analysis and real-time RT-PCR) and protein abundance (Western blot analysis) of P2Y2-R, with hydrated rats showing significantly higher levels compared with dehydrated rats. Sequential hydration and dehydration experiments also revealed that the regulated expression profiles of P2Y2-R mRNA and protein are discordant. Conversely, the expression of V2-R mRNA remained unaltered during hydration and dehydration. Because virtually all renal cells release ATP in a regulated fashion, the observed alterations in P2Y2-R expression in the inner medulla in hydrated and dehydrated states may constitute a novel mechanism of purinergic modulation of IMCD function.

purinergic; V2; cAMP; prostanoids; nucleotides

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salt, and urea by IMCD and thus may constitute a potent AVP-independent regulatory mechanism.

In rats subjected to water loading or hydration, urine osmolality and circulating levels of AVP are very low, similar to the condition of compulsive water drinking seen in humans. This is associated with a decreased protein abundance and/or altered subcellular localization of the AVP-regulated water channel aquaporin-2 (AQP2) in the inner medulla (38). Conversely, water deprivation or thirsting results in elevated urine osmolality and circulating levels of AVP and is associated with an increased protein abundance and apical membrane targeting of AQP2 in inner medulla (38). However, the roles and contributions of agents other than AVP in the regulation of water transport in physiological conditions of hydration and water deprivation have not been fully explored.

Because agonist stimulation of the P2Y2 receptor in IMCD results in a decrease in AVP-induced water flow, we hypothesized that alterations in the expression and/or activity of the P2Y2 receptor in IMCD may occur in conditions of hydration and dehydration. In this communication, we examined the mRNA expression and protein abundance of the AVP-regulated collecting duct water channel AQP2 and the P2Y2 receptor, as well as the vasopressin V2 receptor in renal inner medulla, using two different protocols of hydration and dehydration. We document that hydration and dehydration of rats indeed result in marked alterations in the mRNA expression and protein abundance of P2Y2 receptor in the inner medulla. We discuss the physiological implications of the observed alterations in P2Y2 receptor expression in hydrated and dehydrated states.

MATERIALS AND METHODS

Experimental animals. The animal experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committees of University of Utah, Veterans Administration Salt Lake City Health Care System, Salt Lake City, Utah, and the University of Cincinnati Medical Center, Cincinnati, Ohio. Specific pathogen-free male Sprague-Dawley rats, weighing 200–250 g (Harlan, Indianapolis, IN), were housed two or three per cage in the respective animal facilities, which are accredited by the American Association for Accreditation of Laboratory Animal Care and assured by the US Public Health Service. The rats were maintained in pathogen-free state for Accreditation of Laboratory Animal Care and assured by the US Public Health Service. The rats were acclimated to the housing conditions for about a week before experimental procedures were conducted.

Hydration and dehydration protocols. For hydration and dehydration of rats, we adapted two different protocols as shown in Table 1. Protocol I is similar to the widely used and described protocol (5, 7, 16, 26, 37, 38), except that we included a normal group to the hydrated and dehydrated groups. Protocol II is a modification of protocol I without a prehydration period.

Protocol I. Rats destined for hydration or dehydration were prehydrated by providing 600 mM sucrose solution as the sole drinking fluid for the first 48-h equilibration period. At the end of the first 48-h period, half the animals were randomly chosen for a thirsted or dehydration group and were deprived of water for the next 48 h, whereas the second half continued to receive sucrose water for another 48 h to ensure that they were well hydrated or water loaded. Because the rats in any colony may be at different levels of hydration, the 48-h prehydration period in this protocol ensures equal baseline hydration status before the experimental period of hydration or dehydration. Another group of rats, designated as normal, received only tap water ad libitum (Table 1).

Protocol II. Rats were subjected directly to either hydration by sucrose water loading or dehydration by deprivation of water for 48 h without prehydration. A normal group of rats received only tap water ad libitum (Table 1).

It should be noted that the group designated as “normal” is not an optimal control group for the dehydrated or hydrated groups for the following reasons. Based on natural variation in physiology, individual variation in drinking habits, and animal housing conditions (e.g., variations in the ambient temperature and relative humidity; number of rats per cage), the normal group actually represents a spectrum of hydration states and does not really serve as “controls” for this study. Despite these limitations, we included the normal group in our study as it may indicate the direction, if not the magnitude, of changes in the gene expression and protein abundance in the dehydrated and hydrated groups.

Collection and analysis of urine samples. Urine samples were collected from rats by housing them in individual plastic metabolic cages during the last 24 h of experimental period, just before euthanasia. In some experiments, 24-h urine samples were collected from rats before the commencement of experimental procedures to compare baseline urine parameters in the population of rats used. After collection, volumes of 24-h urine samples were noted, and aliquots of urine samples were centrifuged at 10,000 g for 5 min to remove suspended particles, and the supernatants were used to measure osmolality by either freezing-point depression method (Microosmometer model 3300, Advanced Instruments, Norwood, MA) or vapor pressure method (Wescor model 5100C, Wescor, Logan, UT).

Euthanasia and collection of tissue samples. At the end of experimental period, animals were euthanized by a pentobarbital sodium overdose, and both kidneys were removed rapidly. The kidneys were chilled in ice-cold phosphate-buffered saline, and inner medullas were dissected on ice, flash-frozen in liquid nitrogen, and then stored at −85°C until analyzed.

Northern blot analysis. Northern hybridization was performed as described previously (13, 14). Briefly, total RNA was extracted from inner medullas, using Tri-Reagent (Molecular Research Center, Cincinnati, OH) as per manufacturer’s instructions. Twenty micrograms of total RNA from each rat were size fractionated on 1% formaldehyde agarose gel in 10× MOPS buffer for 4.5 h at 96 V. Equality of sample loading was determined by matching the density of the 28S rRNA band visualized by ethidium bromide. RNA was transferred to Hybond N+ nylon membranes overnight using capillary transfer in 10× SSC and then ultraviolet cross linked. The total RNA blot was prehybridized in 15 ml of ExpressHyb (Clonetech) for 30 min at 65°C, hybridized with 1.5 × 106 cpm/ml of 32P random-labeled (High Prime...
Table 2. Nucleotide sequences of the primer pairs used in PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Ta , °C</th>
<th>Amplicon Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>NM_017008</td>
<td>975–997</td>
<td>1374–1396</td>
<td>57</td>
<td>421</td>
</tr>
<tr>
<td>P2Y2</td>
<td>NM_017255</td>
<td>1270–1293</td>
<td>1376–1399</td>
<td>60</td>
<td>129</td>
</tr>
<tr>
<td>V2 Receptor</td>
<td>NM_019136</td>
<td>626–645</td>
<td>692–711</td>
<td>58</td>
<td>85</td>
</tr>
</tbody>
</table>

Ta, annealing temperature.

DNA labeling with Roche Applied Science) rat P2Y2 receptor or AQP2 partial cDNA probes for 2 h at 65°C. Membranes were washed twice at room temperature for 15 min each in 2× SSC and 0.05% SDS, then 2× at 50°C for 15 min each in 0.1× SSC and 1% SDS. The membranes were stripped by boiling in 0.5% SDS for 30 min and then probed with 32P-labeled cDNA probe for L32. Membranes probed with AQP2, P2Y2, or L32 were exposed to X-ray film (MS film, Kodak) overnight at −70°C with a Kodak MS intensifying screen. X-ray images were digitized, and band densities were determined by using ImageQuant software (Molecular Dynamics, Sunnyvale, CA) or Un-Scan-It (Silk Scientific, Orem, UT) software. Band densities were also quantified by exposing the membranes to phosphor screen and then scanning in a Storm 840 scanner (Molecular Dynamics) and analyzed using ImageQuant software. P2Y2 and AQP2 mRNA expression was normalized to L32 expression levels. The preparation and characterization of cDNA probes for the P2Y2 receptor and AQP2 have been previously described (13, 14).

Quantitative real-time RT-PCR assays. Quantitative real-time RT-PCR was carried out according to methods established in our laboratories (9, 23). Total RNA from inner medullas was isolated using the TRIzol method (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations. The extracted total RNA was further purified using RNeasy Mini Columns with DNase I treatment (Qiagen, Valencia, CA) to remove traces of genomic DNA present in samples. cDNA was made by reverse transcribing 2.5 μg total RNA using oligo (dT) priming and Superscript Reverse Transcriptase II (Invitrogen) according to the manufacturer’s instructions. cDNA was quantified using Smart Cycler II System (Cepheid, Sunnyvale, CA) and SYBR Green for detection. Each PCR reaction contained the following final concentrations: 1× buffer (20 mM Tris-HCl, pH 8.3), 50 mM KCl, 3 mM MgCl2, 0.3 μM forward primer, 0.3 μM reverse primer, 1× additive reagent (0.2 mg/ml bovine serum albumin, 150 mM trehalose, 0.2% Tween 20), 0.25× SYBR Green, 1.5 U Platinum Taq polymerase, 200 μM each dNTPs, 1 μl DNA, and H2O to bring the final volume to 25 μl. Table 2 shows the sequences of the primer pairs used in this study. The cDNA was amplified according to the following steps: 1) 95°C for 2 min; 2) 95°C for 12 s; 3) 58 to 62°C for 15 s (optimized for each primer pair); 4) 72°C for 20 s; and 5) 85°C for 6 s to detect SYBR Green (nonspecific products melt at <85°C, therefore are not detected). Steps 2-5 were repeated for an additional 39 cycles, while at the end of the last cycle temperature was increased from 60 to 95°C (0.2°C/s) to produce a melting curve.

Standard curves were generated using serial dilutions of plasmid templates at the following concentrations: 107, 106, 105, 104, and 103 copies for reaction. PCR reactions were considered valid only if the amplification was linear, i.e., r2 ≥ 0.98 and amplification efficiency was ≥95%. Plasmid templates for quantitative real-time RT-PCR were made by cloning PCR products into the pGEMT plasmid vector (Promega, Madison, WI). Gene copy number per nanogram of DNA was calculated using the size of PCR product plus vector base pairs, while the optical density (O.D.) of plasmid template at 260 nm was used to measure concentration. The expression of P2Y2 and AQP2 gene was computed relative to the expression of the housekeeping gene GAPDH. The validity of the housekeeping gene GAPDH was verified by comparing the expression in copies per microgram of RNA among hydrated, dehydrated, and normal groups within the two protocols.

Preparation of tissue samples and Western blot analysis. Tissue samples were prepared and immunoblotted by the methods reported earlier (6, 14). Briefly, whole tissue homogenates were prepared by homogenizing tissue samples in homogenizing buffer containing protease inhibitors, and protein concentration was determined by BCA Protein Reagent (Pierce, Rockford, IL) and then solubilized in Laemmli sample buffer at 60°C for 20 min. A semiquantitative immunoblotting approach was used to assess the alterations in protein levels as described previously (6, 14). Equality of protein loading was checked by running loading gels and staining the separated proteins with Coomassie blue (Gel Code Blue, Pierce). The membranes were probed with affinity-purified polyclonal antibodies to P2Y2 receptor (L246) or AQP2 (GN-762). Generation, purification, and characterization of these antibodies were described previously (13, 14). Sites of antigen-antibody reaction were visualized by chemiluminescence (SuperSignal Substrate; Pierce) and captured on light-sensitive imaging film (Kodak X-Omat AR or Kodak Biomax ML). Films showing optimal gray scale were digitized either by scanning on a flat-bed scanner or by photographic imaging with a high-pixel digital camera. Relative band densities were quantitated using ImageQuant (Molecular Dynamics) or Un-Scan-It (Silk Scientific) software. Densitometry results are reported as volume integrated values expressed as percentage of the mean values in normal rats (100%).

Statistical analysis. Values are expressed as means ± SE. Data were analyzed by ANOVA followed by assessment of differences between the means of the groups by Tukey-Kramer’s multiple comparison test or Bonferroni’s multiple comparison test. Where applicable, data were also analyzed by unpaired Student’s t-test or non-parametric methods (Mann-Whitney U-test). P < 0.05 was considered significant. The statistical analyses were performed on a PC using the GraphPad InStat Version 3.0 software package (GraphPad Software, San Diego, CA).

RESULTS

Figures 1 to 5 show the results obtained from protocol I, where rats destined for dehydration and hydration were subjected to a 48-h prehydration period. Figures 6 to 10 show the results obtained from protocol II, where rats were directly subjected to dehydration and hydration without a 48-h prehydration period.

![Fig. 1. Urine output and osmolality in normal, dehydrated, and hydrated rats of protocol I. A: 24-h urine volumes. B: urine osmolarities. Data from 2 or 3 experiments were pooled, and values are expressed as means ± SE. A: P < 0.001 normal vs. hydrated group; P < 0.001 dehydrated vs. hydrated groups. B: P < 0.001 normal vs. dehydrated or hydrated groups; P < 0.001 dehydrated vs. hydrated groups. All statistical comparisons were made by ANOVA.](http://ajprenal.physiology.org/ by 10.220.33.1 on June 24, 2017)
Urine parameters in animals of protocol I. Figure 1A shows urine outputs and Fig. 1B shows urine osmolalities in normal, dehydrated, and hydrated groups of protocol I collected during the final 24 h of the experimental period. As shown in the Fig. 1, the dehydrated rats had low output of concentrated urine, whereas the hydrated rats had high output of dilute urine. The differences in the measured parameters among the three groups were large and significant, except for the urine volumes in normal vs. dehydrated groups.

AQP2 water channel and P2Y2 receptor mRNA expression in animals of protocol I. Figure 2 shows expression of mRNA of AQP2 water channel and P2Y2 receptor, as well as that of L32, a housekeeping gene in inner medullas of animals of protocol I, as assessed by Northern hybridization. Densitometry of the bands revealed that normalized AQP2 mRNA expression was significantly lower in the hydrated group compared with the dehydrated group (P < 0.001; Fig. 2B). The AQP2 mRNA levels were also significantly lower in the hydrated group compared with the normal rats (P < 0.05). However, the dehydrated group showed only a modest increase compared with the normal groups (112%), which did not attain statistical significance. Normalized mRNA expression of P2Y2 receptor (Fig. 2C) was significantly lower in the dehydrated rats compared with the hydrated group (P < 0.05) or normal rats (P < 0.05). Thus hydration and dehydration resulted in significant changes in the expression of P2Y2 receptor mRNA, which were in opposite direction compared with the changes in the expression of AQP2 mRNA. Because AQP2 expression is a reflection of cellular cAMP levels, we further compared the AQP2/P2Y2 receptor ratio in the normal, dehydrated, and hydrated rats. Because the mRNAs of these two molecules were determined by hybridization on the same membrane, these were not normalized to the expression of L32 gene. As shown in Fig. 2D, the AQP2/P2Y2 receptor ratio is markedly altered in dehydrated rats compared with hydrated rats.

Figure 3 shows a comparative picture of the AQP2/P2Y2 receptor ratios in normal, dehydrated, and hydrated rats in the representative data from one set of experiments shown in Fig. 2D (n = 4) vs. summary data from two sets of experiments.

Fig. 2. Expression of aquaporin-2 (AQP2) water channel, P2Y2 receptor, and L32 mRNA in inner medullas of normal, dehydrated, and hydrated rats of protocol I, as determined by Northern hybridization. A: representative autoradiographic profiles from 1 experiment. Autoradiographs were digitized and relative band densities were determined. AQP2 and P2Y2 receptor mRNA band densities in each animal were normalized to the corresponding L32 band densities. Normalized values are expressed as means ± SE for each group, as percentage of the mean values seen in the normal group. B: normalized AQP2 mRNA expression in different groups presented in A. *Significantly different from the other 2 groups, P < 0.05 or better by ANOVA. C: normalized P2Y2 receptor mRNA expression in different groups presented in A. *Significantly different from the normal group, P < 0.05 by ANOVA or hydrated group, P < 0.05 by unpaired Student’s t-test. D: AQP2/P2Y2 mRNA expression ratio in different groups presented in A, expressed as percentage of the normal group. *Significantly different from the other 2 groups, P < 0.01 by ANOVA.

Fig. 3. Comparison of AQP2/L32 mRNA expression ratio in representative data obtained from 1 set of experiment vs. summary data obtained from 2 sets of experiments in protocol I. The hatched bars show AQP2/P2Y2 mRNA expression ratio in the representative data (n = 4; same data as in Fig. 2), as determined by densitometry on digitized autoradiographs. The filled bars show AQP2/P2Y2 mRNA expression ratio in the summary data from 2 sets of experiments (n = 7), as determined by phosphorimaging. Note the striking similarities of the data obtained from 2 different sets of experiments using 2 different methods of quantitation. *Significantly different from the other 2 groups, P < 0.01 by ANOVA.
(n = 7). Furthermore, the representative data were processed by densitometry of digitized autoradiographs, whereas the summary data were obtained by PhosphorImaging System. Despite this difference in analytic methods, as shown in the Fig. 3, the alterations in the AQP2/P2Y2 receptor ratio in normal, dehydrated, and hydrated rats are strikingly similar in both the representative and summary data sets.

Figure 4 shows mRNA expression of P2Y2 receptor in inner medullas of normal, dehydrated, and hydrated rats of protocol I, as assessed by real-time RT-PCR approach. The expression of the housekeeping gene GAPDH did not vary significantly among hydrated, dehydrated, and normal groups and therefore was a valid housekeeping gene. The real-time RT-PCR data showed that the mean value of P2Y2 receptor mRNA in dehydrated group was significantly lower (P < 0.02) from that of the hydrated group. The mean values in these two groups showed a 2.5-fold difference. These values, however, were not statistically significant from the mean values in normal animals, mostly due to a large variation in expression observed in that group.

AQP2 water channel and P2Y2 receptor protein abundances in animals of protocol I. Figure 5 shows protein abundances of the AQP2 water channel and P2Y2 receptor in inner medullas of animals of protocol I, as assessed by semiquantitative immunoblotting. In AQP2 immunoblots, the 29-kDa band corresponds to unglycosylated form, whereas the smear from 35 to 50 kDa represents various glycosylated forms of the protein (26). As shown in Fig. 5A, the combined mean densities of the two AQP2 protein bands in hydrated group were significantly lower compared with the normal or dehydrated groups. However, the mean densities of these bands in dehydrated group were not different from the normal rats.

Figure 5 also shows protein abundance of the P2Y2 receptor in inner medullas of normal, dehydrated, and hydrated rats of protocol I. As characterized by us previously, our P2Y2 receptor antibody consistently identifies two sets of immunoreactive bands in kidney and lung (13). Both the 47- to 50-kDa migrating bands and the 105-kDa band appear to be specific, as these were ablated by preadsorption of the antibody with the immunizing peptide (13). The lower set of bands at ~47–50 kDa correspond to glycosylated forms of the receptor protein.

The nature of the upper band ~105 kDa is not known but may represent a dimer of the receptor protein, or a covalent complex of the receptor with another membrane and/or receptor-associated protein, and/or alternatively processed form of the receptor. As depicted in Fig. 5, the protein abundance of the P2Y2 receptor in inner medulla of dehydrated rats was altered by dehydration and hydration of the rats. Densitometry revealed that the combined mean densities of the P2Y2 receptor protein bands were significantly higher in the dehydrated group compared with normal rats and lower compared with the hydrated group. Although the P2Y2 receptor protein abundance in dehydrated group was higher compared with the normal rats, the pattern of
changes in the dehydrated vs. hydrated groups is similar to the mRNA expression pattern (Fig. 2C).

Urine parameters in animals of protocol II. Figure 6A shows urine outputs and Fig. 6B shows the corresponding urine osmolalities in normal, dehydrated, and hydrated groups of protocol II determined collected during the final 24 h of the experimental period. As shown in the Fig. 6, the dehydrated rats had low output of concentrated urine, whereas the hydrated rats had high output of dilute urine. The differences in the measured parameters among these three groups were large and significant as shown in the figure caption. Furthermore, the mean values of urine volumes in normal groups of protocol I (Fig. 1A) and protocol II (Fig. 6A) did not differ (11.9 ± 1.1 vs. 10.6 ± 0.9 ml/24 h). The mean values of urine volumes of dehydrated group in protocol I (Fig. 1A) were slightly higher compared with the dehydrated group in protocol II (Fig. 6A; 2.6 ± 0.3 vs. 1.9 ± 0.3 ml/24 h; P > 0.05). However, the mean urine output of hydrated group in protocol I (96 h; Fig. 1A) was about twofold higher compared with the mean urine output of hydrated group in protocol II (48 h; Fig. 6A; 46.5 ± 4.6 vs. 22.1 ± 2.9 ml/24 h; P < 0.001) consistent with the duration of hydration (96 vs. 48 h).

P2Y2 receptor mRNA and protein expression in animals of protocol II. Figure 7 shows mRNA expression of the P2Y2 receptor in inner medullas of normal, dehydrated, and hydrated rats of protocol II, as assessed by real-time RT-PCR approach. The real-time RT-PCR data showed that the mean value of P2Y2 receptor mRNA in dehydrated group was significantly lower (P < 0.01) compared with that of the hydrated group. The mean values in these two groups showed a 2.2-fold difference. These values, however, were not statistically significant from the mean values in normal rats, mostly due to a large variation of expression observed in that group. Thus the magnitude of the difference in P2Y2 receptor mRNA expression in dehydrated and hydrated groups of protocol II is comparable to the one observed in dehydrated and hydrated groups of protocol I (Fig. 4), using the same approach of real-time RT-PCR.

AQP2 water channel and P2Y2 receptor protein abundances in animals of protocol II. Figure 8 shows protein abundances of
the AQP2 water channel in inner medullas of normal, dehydrated, and hydrated rats of protocol II, as assessed by semi-quantitative immunoblotting approach. The combined mean densities of the two bands of AQP2 protein were increased to 267% in dehydrated and decreased to 34% in hydrated groups compared with normal rats. Due to a large variation in protein abundance observed in normal rats as well as the experimental groups, these values were not significantly different from those of the normal group. However, the difference in the mean values of dehydrated vs. hydrated group attained significance (\( P < 0.03 \)).

Figure 9 shows protein abundances of the P2Y2 receptor in inner medullas of normal, dehydrated, and hydrated rats of protocol II. The combined mean densities of the P2Y2 receptor protein bands in the hydrated group showed more than a threefold increase compared with the corresponding normal or dehydrated groups. Thus, there is a large increase in the protein abundance of P2Y2 receptor in hydrated rats. It is interesting to note that in contrast to dehydrated group of protocol I (Fig. 5), the P2Y2 receptor protein abundance in dehydrated group of protocol II (Fig. 9) did not show an increase but actually decreased compared with the normal rats. In this respect, the results obtained for the dehydrated group in protocol I differed from those of protocol II.

V2 receptor mRNA expression in protocol II. Finally, to verify that the observed alterations in the mRNA expression of the P2Y2 receptor in inner medullas of dehydrated and hydrated groups of protocol II were not due to generalized alterations in the expression of some, if not all, membrane receptors in medullary collecting duct, we determined the mRNA expression of vasopressin V2 receptor in these groups by real-time RT-PCR. As shown in Fig. 10, the mRNA expression of the V2 receptor did not show any difference between the dehydrated and hydrated groups of protocol II, although the mean values in these two groups are numerically, but not statistically, low compared with the control group.

DISCUSSION

Pharmacological and molecular approaches localized P2Y2 receptor in the IMCD of the rat (5, 13). Our previous studies demonstrated that the agonist activation of the P2Y2 receptor in rat IMCD (1) reversibly downregulates AVP-stimulated osmotic water permeability by decreasing cellular cAMP levels by a protein kinase C-dependent mechanism (12) and (2) results in enhanced production and release of PGE2 in a COX-1-dependent manner (40). PGE2 is a major prostanoid in the kidney and it interacts with four G protein-coupled E-prostanoid receptors designated EP1, EP2, EP3, and EP4. Through these receptors, PGE2 modulates renal hemodynamics and salt and water excretion (2). Although the functional antagonism of PGE2 on AVP-stimulated collecting duct water permeability in IMCD is known for a while (10, 24), only recently have the cell biological mechanisms of this antagonistic effect been unraveled. Using ex vivo preparations of renal medulla, Zelenina et al. (41) demonstrated that agonist stimulation of the EP3 prostanoid receptor in IMCD causes retrieval of AQP2 water channels from the apical membrane, thus reducing the abun-
dance of AQP2 protein in the apical membrane, the rate-limiting barrier in the transepithelial water movement in the collecting duct. In view of these potent interactions among vasopressin, purinergic, and prostanoid systems in the IMCD, we hypothesized that conditions such as hydration and dehydration, where the biology of collecting duct is altered, may be associated with changes in the expression and/or activity of P2Y2 receptor, which can lead to modulation of collecting duct water permeability.

In this communication, we demonstrate that physiological conditions such as dehydration and hydration, which are associated with changes in medullary collecting duct biology, indeed show significant alterations in mRNA expression and protein abundance of P2Y2 receptor. Our results clearly document that the mRNA expression and protein abundance of the P2Y2 receptor in the inner medullas of hydrated rats are consistently higher compared with the dehydrated rats in two different protocols. We also document that the observed changes in the expression of P2Y2 receptor mRNA are in opposite direction to the changes seen in the mRNA expression of AVP-regulated AQP2 water channel of the collecting duct. Furthermore, because such changes are not observed in the mRNA expression of the vasopressin V2 receptor, it appears that the observed changes in P2Y2 receptor mRNA expression are specific and are not reflective of generalized alterations in other G protein-coupled receptors in the medullary collecting duct in conditions of hydration and dehydration. To complement these molecular observations, we also obtained functional data showing that P2Y2 receptor-stimulated PGE₂ release by the IMCD is significantly higher in the hydrated rats compared with the dehydrated or normal rats. This is associated with marked differences in the urinary excretion of PGE₂ metabolite in the hydrated and dehydrated rats (34, 35). The decrease in P2Y2 receptor mRNA expression in dehydrated rats compared with hydrated rats was consistent between both protocols. Unlike the consistent decrease in P2Y2 receptor mRNA expression observed, however, P2Y2 receptor protein abundance was significantly increased in dehydrated rats compared with the normal rats in protocol I, where the rats were prehydrated for 48 h with sucrose water before thirsting. Rats that were not prehydrated with sucrose water for 48 h before thirsting (protocol II) showed the opposite trend, with >50% reduction in the amount of P2Y2 receptor protein in the dehydrated group compared with normal rats. These reciprocal changes in P2Y2 receptor protein exist in the presence of consistent and comparable urinary parameters of dehydration (decreased volume and increased osmolality) in both protocols. Hence, this apparent dissociation is perhaps due to the inherent nature of the protocols used. Furthermore, from the data presented in protocol II, where there was no prehydration period, it appears that compared with normal rats, the changes in mRNA expression are more sensitive to dehydration, whereas the changes in protein abundance are more prominent in hydrated rats, indicating a differential regulation of P2Y2 receptor mRNA expression and protein abundance. These aspects obviously need further studies to investigate the half-life, stability, and turnover of P2Y2 receptor mRNA and protein and subcellular distribution of P2Y2 receptor protein in IMCD under the conditions of hydration and dehydration as laid out in these two protocols.

Although there are marked and significant differences in the protein abundances of AQP2 in dehydrated vs. hydrated rats, yet in our series it appears that compared with normal rats, changes in AQP2 protein abundance are more sensitive to hydration than dehydration. While interpreting these data, one should take into account that the water permeability of collecting the duct is determined by not just the protein abundance but also by membrane targeting of AQP2 as well as medullary interstitial osmotic gradients (3, 15, 19).

Our real-time RT-PCR data indicate that V2 receptor mRNA expression is unchanged in dehydrated and hydrated rats. The consistent expression of V2 receptor mRNA excluded the possibility that the observed changes in P2Y2 receptor expression are not due to generalized changes in gene expression under these conditions. For lack of a good quality antibody to V2 receptor protein, we could not determine the protein abundance of this receptor.

Finally, three potential mechanisms for the observed changes in P2Y2 receptor expression in dehydrated and hydrated rats are readily apparent. Altered circulating levels of AVP, known to accompany changes in hydration status, may regulate gene expression directly through transcriptional/translational control via intracellular signaling. Alternatively, AVP may indirectly influence P2Y2 receptor expression through changes in medullary interstitial osmolality. Or, a third, yet unidentified mechanism may be operating. Our preliminary data on chronically dDAVP (a V2 receptor-specific analog of AVP)-infused rats showed that the P2Y2 receptor mRNA expression and protein abundance in the inner medulla were decreased to ~50% of saline-infused control rats (17). This may be due to a direct effect of dDAVP on the medullary collecting duct or indirectly through increased medullary interstitial osmolality. Currently, we are examining these possibilities in primary cultures of IMCD cells, where, unlike in vivo conditions, the direct effect of dDAVP and the indirect effect of osmolality can be tested separately.

In conclusion, our current study on altered expression of P2Y2 receptor mRNA and protein perhaps constitutes the first molecular evidence for potential non-vasopressin mechanisms that may modulate collecting duct function in physiological conditions of hydration and dehydration.

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