

Hypokalemia-induced Downregulation of Aquaporin-2 Water Channel Expression in Rat Kidney Medulla and Cortex

David Marples, Jørgen Frøkiær,* Jens Dørup, Mark A. Knepper,‡ and Søren Nielsen

Department of Cell Biology, Institute of Anatomy, University of Aarhus, DK-8000 Aarhus, Denmark; *Department of Clinical Physiology, Aarhus University Hospital and Institute of Experimental Clinical Research, University of Aarhus, DK-8000 Aarhus, Denmark; and ‡National Heart, Lung, and Blood Institute, National Institute of Health, Bethesda, Maryland 20892

Abstract

Prolonged hypokalemia causes vasopressin-resistant polyuria. We have recently shown that another cause of severe polyuria, chronic lithium therapy, is associated with decreased aquaporin-2 (AQP2) water channel expression (Marples, D., S. Christensen, E.I. Christensen, P.D. Ottosen, and S. Nielsen, 1995. *J. Clin. Invest.*, 95: 1838–1845). Consequently, we studied the effect in rats of 11 days' potassium deprivation on urine production and AQP2 expression and distribution. Membrane fractions were prepared from one kidney, while the contralateral kidney was perfusion-fixed for immunocytochemistry. Immunoblotting and densitometry revealed a decrease in AQP2 levels to $27 \pm 3.4\%$ of control levels ($n = 11$, $P < 0.001$) in inner medulla, and $34 \pm 15\%$ of controls ($n = 5$, $P < 0.05$) in cortex. Urine production increased in parallel, from 11 ± 1.4 to 30 ± 4.4 ml/day ($n = 11$, $P < 0.01$). After return to a potassium-containing diet both urine output and AQP2 levels normalized within 7 d. Immunocytochemistry confirmed decreased AQP2 labeling in principal cells of both inner medullary and cortical collecting ducts. AQP2 labeling was predominantly associated with the apical plasma membrane and intracellular vesicles. Lithium treatment for 24 d caused a more extensive reduction of AQP2 levels, to $4 \pm 1\%$ of control levels in the inner medulla and $4 \pm 2\%$ in cortex, in association with severe polyuria. The similar degree of downregulation in medulla and cortex suggests that interstitial tonicity is not the major factor in the regulation of AQP2 expression. Consistent with this furosemide treatment did not alter AQP2 levels. In summary, hypokalemia, like lithium treatment, results in a decrease in AQP2 expression in rat collecting ducts, in parallel with the development of polyuria, and the degree of downregulation is consistent with the level of polyuria induced, supporting the view that there is a caus-

ative link. (*J. Clin. Invest.* 1996. 97: 1960–1968.) Key words: aquaporins • water channels • vasopressin • nephrogenic diabetes insipidus • polyuria

Introduction

Hypokalemia is a common, and sometimes life-threatening, electrolyte abnormality, that can arise from a wide variety of causes (1). Prolonged hypokalemia causes substantial morphological change in kidney ultrastructure, and is associated with renal functional abnormalities, including a vasopressin-resistant decrease in urinary concentrating ability (1). The rat provides a good animal model of these effects, with changes similar to those seen in human kidney (2).

Body water balance is regulated by vasopressin, which increases the water permeability of the collecting duct, allowing increased reabsorption of water down an osmotic gradient. It has recently been demonstrated that aquaporin-2 (AQP2) is the vasopressin-regulated water channel, and is found only in the collecting duct (3–5). Furthermore, it has been demonstrated that genetic defects in AQP2 result in severe nephrogenic diabetes insipidus (6). The acute vasopressin-induced increase in water permeability of the collecting duct is caused by the transfer of water channels from intracellular vesicles to the apical plasma membrane (7–10). There is increasing evidence that vasopressin, in addition to regulating the acute increase in collecting duct water permeability, also plays a longer term role, by modulating the level of AQP2 expression (4, 5, 11). In particular, we have recently shown that chronic lithium treatment, which also induces a vasopressin-resistant polyuria, is associated with a decrease in AQP2 expression in the inner medullary collecting duct of rats (12).

The mechanism by which hypokalemia decreases urinary concentrating ability is not well established (1). While hypokalemia has been associated with increased prostaglandin production (13–15), and inhibitors of prostaglandin synthesis restore the responsiveness of rabbit isolated perfused collecting ducts to vasopressin (14), prostaglandins do not appear to be an important factor in the etiology of hypokalemia-induced polyuria in rats (16). In the rat, there may be a decrease in adenylyl cyclase sensitivity in response to vasopressin (2, 17), thus reducing production of cAMP, the second messenger for vasopressin action. In addition to inhibiting the acute vasopressin-induced increase in water permeability, this decrease in cAMP may also be the messenger causing a decrease in AQP2 expression. Hypokalemia also results in decreased NaCl reabsorption in the thick ascending limb of the loop of Henle, and this will reduce medullary osmolality, and hence the driving force for water reabsorption (18).

Address correspondence to Søren Nielsen, M.D., Ph.D., Department of Cell Biology, Institute of Anatomy, University of Aarhus, DK-8000 Aarhus, Denmark. Phone: 8942 3046; FAX: 8619 8664; E-mail: sn@ana.aau.dk

Received for publication 20 June 1995 and accepted in revised form 14 February 1996.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/96/04/1960/09 \$2.00

Volume 97, Number 8, April 1996, 1960–1968

We have now studied the effect of chronic hypokalemia in rats, to determine whether downregulation of AQP2 is a common feature in a range of drug-induced forms of nephrogenic diabetes insipidus, or is a specific feature of lithium treatment. Furthermore, we have extended our investigation to test whether the downregulation could be due to a decrease in interstitial tonicity caused by washout of the medullary osmotic gradient, rather than a direct effect of a change in responsiveness to vasopressin. We have used two approaches to test this: first, we have examined the effects of lithium and hypokalemia on AQP2 expression to cortical collecting ducts, and second we have used furosemide to abolish the osmotic gradient in the inner medulla. These studies help to determine the etiology of the decrease in expression.

Our results demonstrate that hypokalemia causes a decrease in AQP2 levels in association with the development of nephrogenic diabetes insipidus. Neither the degree of polyuria nor the decrease in AQP2 levels is as great as that seen after lithium treatment, further supporting the view that there is a causative link between AQP2 levels and diuretic status. A downregulation of expression is also seen in the cortex after both hypokalemia and lithium therapy, while furosemide has no effect, suggesting that the decrease in AQP2 levels is not a consequence of a change in the medullary tonicity, but consistent with decreased responsiveness to vasopressin.

Methods

Experimental animals. Male Wistar rats, initially weighing 256 ± 6 grams, were obtained from Møllegaard Breeding Centre Ltd, Denmark. Rats were kept in metabolic cages for at least 48 h before starting a low potassium diet, and had free access to standard rat chow and water. Dietary depletion efficiently causes significant hypokalemia and reduced urinary potassium excretion to $< 10\%$ of control values at day 11 (19). Consequently, rats in the present study were placed on a potassium-deficient diet (C10367, Altromin International) for 11 d. Control rats had free access to standard chow and water throughout. All animals had access to a salt block. Water and food consumption, urine production, and weight were measured daily.

In the experiments performed to investigate the effect of furosemide, rats were kept in metabolic cages for 4 d before the start of treatment. Furosemide, 25 mg in 2.5 ml of saline, was given i.p. at 8 hourly intervals for 4 doses, and animals were killed 2 h after the last dose (i.e., after 26 hours total). Controls were given i.p. saline. Urine output was also measured at 8 hourly intervals during this period. During furosemide treatment, animals had free access to water containing 0.1% KCl and 0.8% NaCl, to avoid salt or potassium depletion.

Tissue preparation. For removal of kidneys, rats were anaesthetized with intraperitoneal sodium pentobarbital, 75 mg/kg. The right kidney was clamped and removed for preparation of crude membrane fractions. The left kidney was then fixed for immunocytochemistry by retrograde aortic perfusion with ice-cold fixative (8% paraformaldehyde in 0.1 M Na cacodylate buffer, pH 7.4) for 3 minutes, as previously described (4, 20).

Preparation of membranes and gel samples. Membrane fractions were prepared as previously described (12). The cortex and inner medulla were dissected from each kidney, minced finely, and homogenized in 10 ml of dissecting buffer (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, pH 7.2 and containing the following protease inhibitors: 8.5 μ M leupeptin, 1 mM phenylmethyl sulfonylfluoride), with five strokes of a motor-driven Potter-Elvehjem homogenizer, at 1250 rpm. This homogenate was centrifuged in a Beckman L8M centrifuge at 4,000 g for 15 min at 4°C. The pellet was rehomogenized with three strokes, and the centrifugation repeated. The supernatants were pooled, and centrifuged at 200,000 g for 1 h. The resultant pellet was

resuspended in approximately 100 μ l of dissecting buffer, and assayed for protein concentration using the method of Lowry. Gel samples (in Laemmli sample buffer containing 2% SDS) were made from this membrane preparation to a final concentration of 1 μ g of protein/ μ l (medulla), and 4 μ g of protein/ μ l (cortex).

Electrophoresis and immunoblotting. Samples prepared from membrane fractions were loaded at 2–10 μ g/lane (inner medulla) or 30 μ g/lane (cortex) onto 12% SDS/PAGE minigels (Biorad Mini Protean II). Proteins were transferred to nitrocellulose paper by electroelution. After blocking for 1 h with PBS-T (80 mM Na_2HPO_4 , 20 mM NaH_2PO_4 , 100 mM NaCl, 0.1% Tween 20, pH 7.5) containing 5% dried skimmed milk, blots were washed with PBS-T (1 \times 15 min + 2 \times 5 min), and incubated overnight at 4°C with an antibody (5, 7, 8, 12) raised against the COOH-terminal 22 amino acids of AQP2 (immune serum, diluted 1:1000). Blots were labeled overnight at 4°C with the same antibody after affinity purification using the immunizing peptide, to increase the specificity of labeling. The affinity-purified antibody was used at a concentration of 0.3 μ g/ml. After washing as above, the blots were incubated with horseradish peroxidase-conjugated secondary antibody (P448 from Dako, Denmark, diluted 1:3000) for 1 h at room temperature. After a final washing, antibody binding was visualized using the ECL (enhanced chemiluminescence) system (Amersham International, U.K.). Controls were made with exchange of primary antibody to antibody pre-absorbed with immunizing peptide (100 ng per 40 ng IgG), or with pre-immune serum (diluted 1:1000). All controls were without labeling. ECL films were scanned using a Hewlett-Packard Scanjet scanner, and quantitated using specially-written software (available upon request). Bands from gels made with serial dilutions of protein from inner medulla, processed as above, were found to be linear over a wide range, as previously described (12). For quantitation of AQP2 expression, ECL exposures were chosen that gave bands in the control samples that were close to the top of the linear range. Parallel gels, stained with Coomassie brilliant blue, were used to ensure that protein loading of lanes was uniform.

Preparation of tissue for immunocytochemistry. Tissue blocks prepared from the kidney inner medulla and cortex were post-fixed for 2 h, and infiltrated with 2.3 M sucrose/2% paraformaldehyde for 30 min. Blocks were mounted on holders, and rapidly frozen in liquid nitrogen. Thin (0.85 μ m) and ultrathin (80 nm) cryosections, cut on a Reichert Ultracut FCS, were incubated with the affinity purified antibody against AQP2 (100–800 ng IgG/ml). The labeling was visualized as previously described (4, 20) using horseradish peroxidase-conjugated secondary antibodies for light microscopy, fluorescein-conjugated secondary antibodies for fluorescence microscopy, or using goat anti-rabbit gold (10 nm particles) for electron microscopy. In double-label fluorescence studies, the vacuolar H^+ ATPase was localized with a mouse monoclonal antibody, kindly supplied by Dr. Steven Gluck (E11, diluted 1:40), which was mixed with the primary antibody against AQP2. In these studies, the labeling was visualized using a rhodamine conjugated swine-anti rabbit antibody (DAKO R156, diluted 1:40) mixed with a fluorescein conjugated goat anti-mouse antibody (DAKO F479, diluted 1:20). Controls using pre-immune serum, antiserum absorbed with excess synthetic peptide (as described above), or omission of primary or secondary antibody, revealed no labeling. Electron micrographs were taken on Philips 208 and 100CM electron microscopes, at an original magnification of 6,000.

Statistics. For densitometry of immunoblots, samples from four to six hypokalemic animals were run on each gel with three to six representative controls. AQP2 labeling in the samples from each hypokalemic animal was calculated as a fraction of the mean control value for that gel. Values are presented in the text as means \pm standard errors. Comparisons between groups were made by unpaired *t* test.

Results

Hypokalemia induces polyuria and polydipsia. During the period on normal diet, urine production averaged 12 ± 1 ml/day.

Table I. Summary of Physiological Parameters, Expressed as Mean ± SE

		n	Control period -1	Experimental period (days)				
				2	4	6	8	10
Water intake (ml/day)	Hypokalemia	11	22±2	23±1	26±2	40±4	39±3	28±2
	Control	6	20±2	21±1	21±2	22±2	22±2	22±2
Urine production (ml/day)	Hypokalemia	11	12±1	13±1	20±2	27±3	29±3	21±2
	Control	6	11±1	11±1	12±2	11±1	11±1	11±1
Potassium excretion (mmol/day)	Hypokalemia	6	2.4±0.2	2.0±0.4	0.3±0.04	0.2±0.02	0.1±0.01	0.1±0.02
	Control	6	2.4±0.1	4.7±0.4	3.6±0.2	4.5±0.3	4.5±0.2	3.8±0.4

After commencing a low potassium diet, urinary excretion of potassium falls precipitously, while water intake and urine production both increase progressively. In the rats on the hypokalemic diet average body weight declined from 249±8 to 241±6 grams, while controls gained an average of 10±5 grams. This data is derived from the animals used for determination of AQP2 levels at day 11. A separate group of animals were used in the investigation of the reversibility of these changes, as shown in Fig. 7.

This rose to a maximum of 29±3 ml/day on day 8 of the potassium-deficient diet. There was a parallel increase in water intake, from 22±2 to 39±3 ml/day. Values were unchanged in control animals. Urinary excretion of potassium decreased rapidly on the potassium-deficient diet, stabilizing at ~5% of control levels. These data are summarized in Table I. The increase in urine output was associated with a modest, but significant impairment of urinary concentrating ability following 12 h water deprivation: urine osmolality rose to 1612±124 mOsm/Kg H₂O in hypokalemic animals, and 2566±195 mOsm/Kg H₂O in controls.

Hypokalemia reduces AQP2 expression in both inner medulla and cortex. Immunoblotting of membrane samples prepared from renal inner medulla (Fig. 1) and cortex (Fig. 2) of control animals revealed two prominent bands at 29 and 35–50 kD, representing the non-glycosylated and glycosylated forms of AQP2 (3, 4), respectively. No labeling was seen with antibody pre-adsorbed with the immunizing peptide (cortex or inner medulla), or with pre-immune serum (inner medulla).

After 11 d on the potassium-free diet, AQP2 expression was substantially reduced in both the inner medulla (Fig. 1) and cortex (Fig. 2). Densitometric quantitation revealed a de-

crease in expression to 27±3% of control levels ($n = 11, P < 0.001$) in inner medulla. The reduction in expression in the cortex of hypokalemic rats was similar (34±15% of control; $n = 5, P < 0.05$). Treatment with lithium for 25 d has previously been shown to reduce AQP2 levels in the inner medulla to 4±1% (12). We now report that 25 d lithium treatment resulted in a comparable decrease of AQP2 in cortex to 4±2% ($n = 5, P < 0.001$). Thus, for both chronic hypokalemia and lithium therapy, the decrease in AQP2 levels found in the cortex was similar to that seen in the inner medulla.

Immunocytochemistry confirms the downregulation of AQP2 during hypokalemia. Immunolabeling of AQP2 on thin (0.85 µm) cryosections of renal inner medulla with horseradish peroxidase- or fluorescein-conjugated secondary antibodies confirmed that there was an overall decrease in AQP2 expression (Fig. 3). These sections also demonstrate that the collecting ducts undergo a marked hypertrophy relative to the controls (compare Fig. 3 a and c with b and d), a change characteristically seen after prolonged hypokalemia (19). A similar pattern was seen in cortex, as shown in Fig. 3, f–j. Both hypokalemia (Fig. 3 i) and lithium treatment (Fig. 3 j) caused a downregulation comparable to that seen in the inner medulla, confirming the results obtained by immunoblotting. Double immunolabeling for the vacuolar H⁺ ATPase (a marker for intercalated

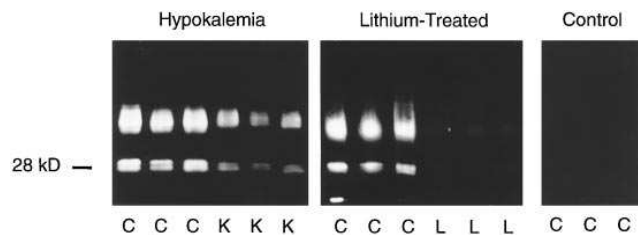


Figure 1. Immunoblots showing the effect of hypokalemia and lithium treatment on AQP2 levels in rat kidney inner medulla. Membrane protein (1 µg) was loaded in each lane, and the blots labeled with antibody against AQP2. The left panel compares expression in three control animals (C), with that in three hypokalemic animals (K). Bands corresponding to 29 and 35–50 kD can be seen in the controls. These bands are also visible in the samples from hypokalemic animals, but are much fainter. After lithium treatment (L) an even more prominent decrease in labeling is seen (center panel). After preabsorption of the primary antibody with excess immunizing peptide no labeling is seen (right panel).

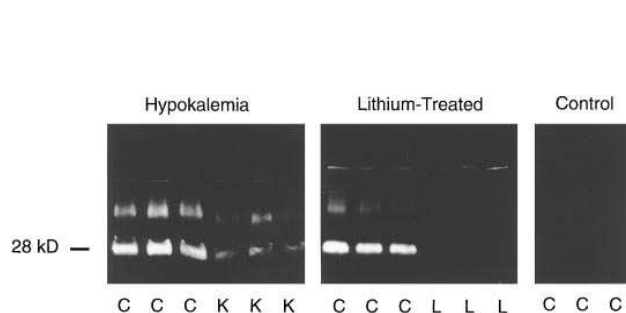
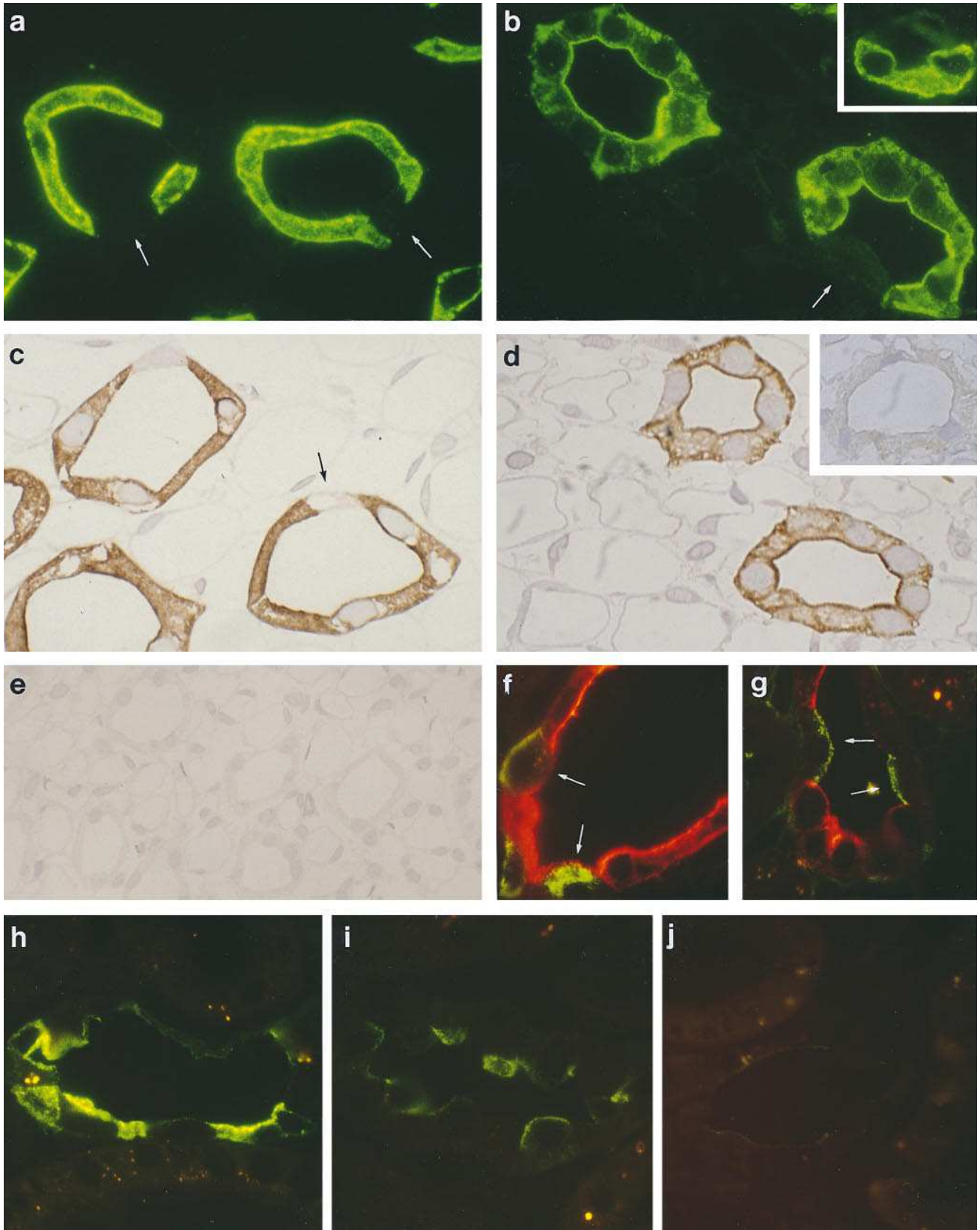


Figure 2. Immunoblots showing the effect of hypokalemia and lithium treatment on AQP2 levels in rat cortex. Membrane protein (30 µg) was loaded in each lane, and blots labeled with affinity-purified antibody against AQP2. Both hypokalemia (K, left panel) and lithium treatment (L, center panel) were associated with a substantial decrease in AQP2 levels, compared with controls (C). After preabsorption of the primary antibody with excess immunizing peptide no labeling is seen (right panel).



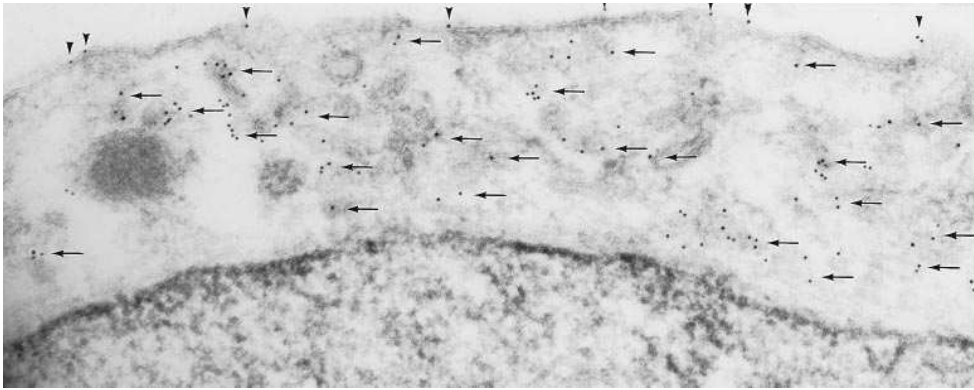


Figure 4. Electron micrograph showing the apical part of a principal cell from an inner medullary collecting duct of a control animal immunogold labeled for AQP2. There is heavy labeling both of the apical plasma membrane (*arrowheads*) and of intracellular vesicles (*arrows*). $\times 68,000$.

cells) and AQP2 (Fig. 3, *f* and *g*) confirmed selective localization of AQP2 to principal cells.

The degree and pattern of labeling varied between animals, with markedly reduced levels (compare Fig. 3, *a* and *b*), while in others the decrease was less extensive (compare Fig. 3, *c* and *d*, and compare 3 *d*, and its inset). Furthermore, in some tubules the labeling of all cells within a tubule was uniform (Fig. 3, *b* and *d*), while in other cases the labeling varied substantially from cell to cell within a single tubule (inset to Fig. 3 *b*). In general, there was prominent labeling of the apical part of the cell suggesting that endogenous vasopressin was still able to cause redistribution of AQP2.

Electron microscopy of immunogold-labeled ultrathin (80 nm) cryosections of inner medulla from hypokalemic rats demonstrated labeling for AQP2 associated with both apical and basolateral plasma membranes, as well as intracellular vesicles (Fig. 5), but at levels lower than that seen in the controls (Fig. 4). The granules characteristically formed during chronic hypokalemia (21, 22), which are believed to represent lysosomal structures (1, 23) were clearly visible. Many of these granules were labeled for AQP2, as shown in Fig. 6.

Return to normokalemia corrects AQP2 downregulation. To examine the reversibility of the effect of hypokalemia, rats were returned to a normal diet for 7 d after an 11-d period on a potassium-deficient diet. Fig. 7 shows the water intake in this group, together with their controls. At the end of the recovery period, water intake was 28 ± 2 ml/day in the experimental animals, and 29 ± 1 ml/day in the controls. No urinary concentrating defect was detected. Consistent with this, AQP2 levels had

returned to normal levels (1.05 ± 0.08 times the level in controls, $n = 6$, n.s.), as shown in Figs. 8 and 9.

Washout of the medullary osmotic gradient does not affect AQP2 levels. We used two approaches to determine whether the loss of the high interstitial tonicity in the inner medulla was a major factor regulating AQP2 expression. First, as described above, we determined AQP2 expression in cortical collecting ducts, which are exposed to much smaller osmotic stimuli. Second, we investigated the effect of the loop diuretic furosemide. This treatment resulted in an increase in urine production from 5 ± 1 to 52 ± 7 ml/day ($n = 12$, $P < 0.001$). In controls, urine output rose to 12 ± 1 ml/day ($n = 12$, $P < 0.01$), reflecting the intraperitoneal fluid load associated with the injections of vehicle. Urine osmolality in the controls was 1230 ± 153 mOsm/Kg H_2O , and fell to 410 ± 15 in the furosemide-treated animals ($n = 12$, $P < 0.001$). AQP2 expression in the furosemide treated animals was not significantly changed, averaging $91 \pm 5\%$ ($n = 9$, n.s.) of the level in controls (Fig. 10). Thus loss of the high medullary interstitial tonicity does not appear to have a major effect on AQP2 expression. This is consistent with the observation that the downregulation in cortical collecting ducts was identical to that seen in inner medullary collecting ducts of both hypokalemic and lithium-treated rats (compare Figs. 1 and 2, Fig. 3).

Discussion

The results presented here demonstrate that chronic potassium depletion causes a substantial decrease in AQP2 expres-

Figure 3. Immunocytochemical localization of AQP2 in $0.85 \mu\text{m}$ cryosections of inner medulla (*a–e*) and cortex (*f–j*). (*a*) Immunofluorescent localization of AQP2 in control inner medulla. Labeling is seen only in the principal cells of collecting ducts, with no labeling of intercalated cells (*arrows*). The apical and (more weakly) the basolateral plasma membrane domains both label, together with intracellular vesicles. (*b*) In hypokalemic animals, the labeling is weaker, but still primarily localized to the apical plasma membrane domain. In some tubules, AQP2 labeling is uniform in all principal cells (*d*), while in other cases there is considerable cell to cell variability (*inset*). Note the characteristic increase in cell height in the hypokalemic animals. (*c* and *d*) Immunoperoxidase labeling of AQP2. In some collecting ducts the decrease in labelling was less marked, while in others (*inset*) it was prominent. (*e*) Control made using nonimmune IgG in place of primary antibody. No labeling is seen. (*f*) Cortical collecting duct in a control animal double labeled using immunofluorescence. AQP2 labeling (rhodamine, *red*) is seen associated with the apical plasma membrane domain and with intracellular vesicles in the subapical region of principal cells. Intercalated cells labeled for the vacuolar H^+ ATPase (fluorescein, *green*) on either their apical (*bottom arrow*) or basolateral (*left arrow*) domains, but showed no labeling for AQP2. (*g*) Cortical collecting duct in a hypokalemic animal double labeled using immunofluorescence as described for panel *f*. Labeling for AQP2 is substantially weaker than seen in the control. (*h–j*) Cortical collecting ducts labeled for AQP2 using fluorescein. In control animals (*h*) strong labeling is seen in the apical part of the principal cells. In hypokalemic animals (*i*), labeling is much reduced, while in lithium treated animals (*j*), labeling for AQP2 is virtually absent. $\times 900$.

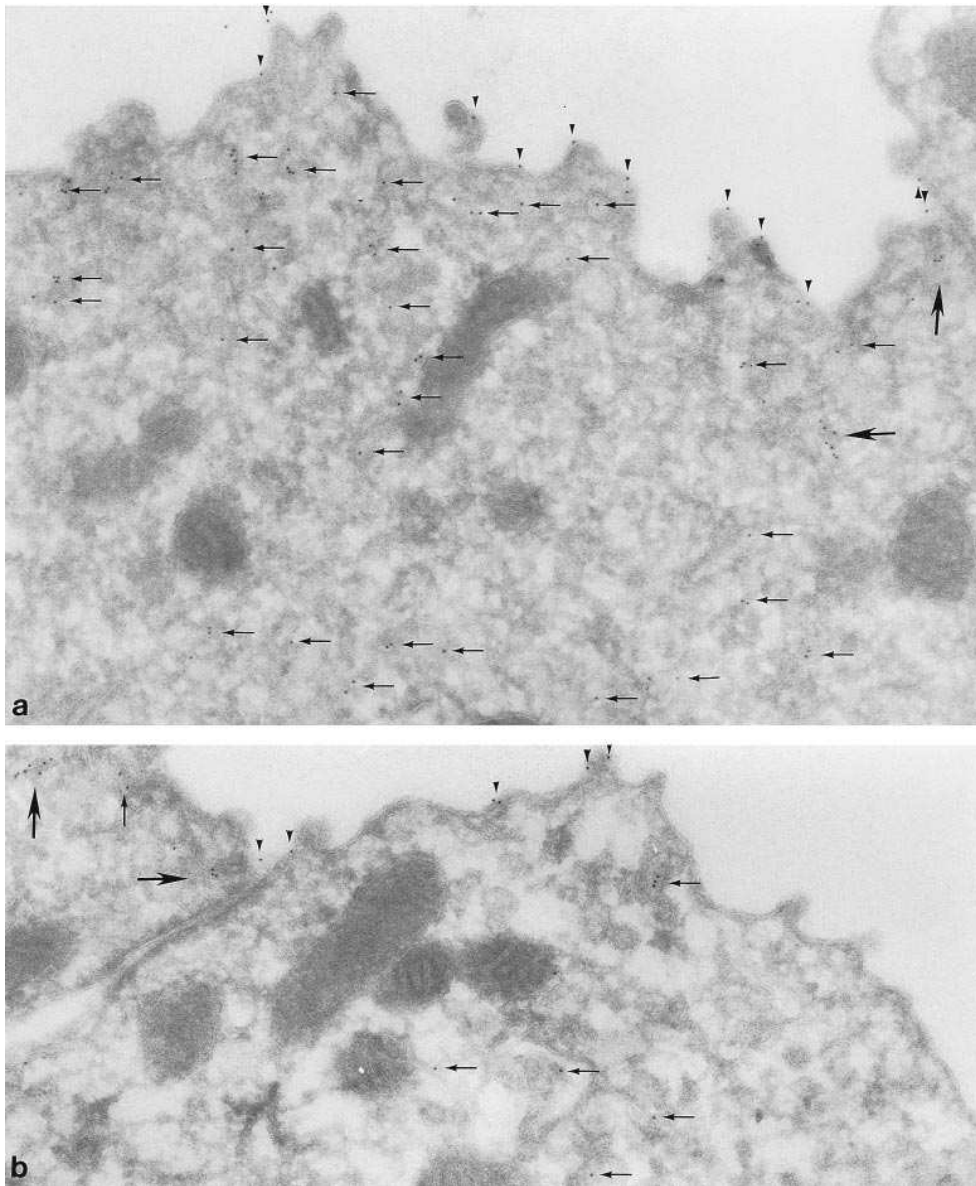


Figure 5. Electron micrographs of the apical region of two adjacent principal cells from a hypokalemic animal. (a) Both the plasma membrane (*arrowheads*) and intracellular vesicles (*arrows*) label for AQP2. Although less label is present than in the control (see Fig. 4), there is considerably more label than seen in an adjacent cell (*b*). For orientation, large arrows indicate the same structures in *a* and *b*. $\times 68,000$.

sion in the renal collecting ducts, in association with the development of polyuria and a urinary concentrating defect. After return to a normal diet, AQP2 expression recovered completely within a week. Physiological parameters also normalized, and no urinary concentrating defect was seen at this time. Thus the AQP2 levels remain correlated with the physiological parameters throughout the experiment. In conjunction with our previously reported findings of similar parallel decreases following lithium treatment (12) or bilateral ureteric obstruction (24), these results strongly suggest that decreased AQP2 levels are likely to be common in a range of acquired forms of nephrogenic diabetes insipidus, and critically involved in the genesis of the concentrating defect associated with these conditions.

It has previously been shown that dehydration (4) or chronic infusion of AVP (5) cause an increase in AQP2 expression in renal inner medulla, while water loading (which would be expected to suppress endogenous vasopressin re-

lease) causes a decrease in AQP2 levels (4), suggesting that there is a long-term modulation of AQP2 expression in response to circulating vasopressin levels. Although the mechanisms by which hypokalemia causes nephrogenic diabetes insipidus are a matter of contention, it appears to be due to a reduction in the production of cAMP (the second messenger for vasopressin action) in response to vasopressin, either by direct inhibition of adenylate cyclase (2, 16, 25) or via increased prostaglandin production (14). Our previous observation that lithium causes a decrease in AQP2 expression could also be explained by this mechanism, as the lithium-induced polyuria is thought to be due to an inhibition of adenylate cyclase (26, 27). This would suggest that the modulatory effect of vasopressin on AQP2 expression is mediated via the same (V_2) receptors that mediate the acute antidiuretic response. However, thirsting caused a more pronounced increase in AQP2 expression than chronic infusion of dDAVP (a V_2 selective vasopressin analogue) in lithium-treated rats (12), suggesting that

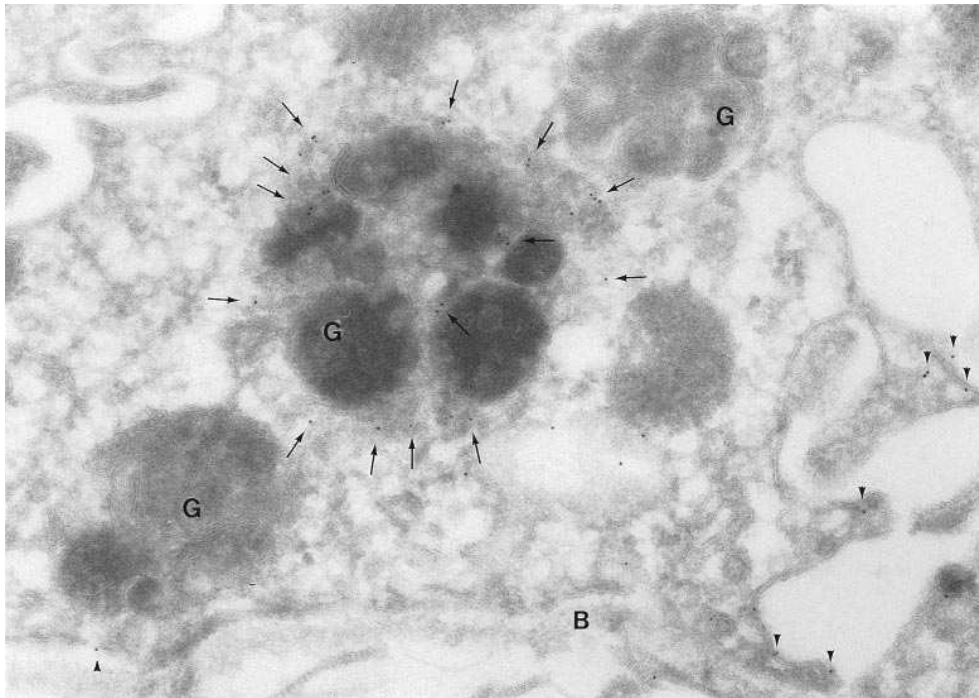


Figure 6. Electron micrograph of the basal part of a principal cell from a hypokalemic animal. Three granules (G) can be seen, of which the middle one is heavily labeled for AQP2 (arrows). The basolateral plasma membrane is also labeled (arrowheads). Basement membrane (B). $\times 68,000$.

additional factors may be involved under these conditions. Other possible signals that could trigger the increase in expression might be a non- V_2 receptor mediated response to vasopressin, or changes in interstitial osmolality. The latter would fit with the inhibition of NaCl transport in the thick ascending limb of the loop of Henle by hypokalemia (1, 18), and/or medullary washout as a consequence of increased urine flow. In order to investigate this possibility, we looked at AQP2 expression in the cortex, where the high blood flow keeps the interstitial osmolality very close to that of the plasma. Both hypokalemia and lithium treatment caused a decrease in AQP2 expression in the cortex essentially identical to that seen in the

inner medulla, suggesting that the tissue osmolality is unlikely to be an important factor. This is confirmed by the experiment with the loop diuretic furosemide, which rapidly ablates the osmotic gradient in the medullary interstitium, yet had no significant effect on AQP2 levels. Thus it appears likely that the reduced AQP2 expression is a result of reduced responsiveness to vasopressin. Furthermore, the studies with furosemide demonstrate that a diuresis per se does not affect AQP2 expression.

A feature of the AQP2 expression in the tissues of hypokalemic animals was the heterogeneity of the degree of downregulation. While labeling in control tissues is uniform, in hypokalemic animals there was variation both between tubules, and between adjacent cells within a single tubule. This is in contrast to the observations in the lithium-treated animals, where expression was uniformly very low. Of course, the extreme downregulation seen after lithium may have masked any variability. The heterogeneity of expression between prin-

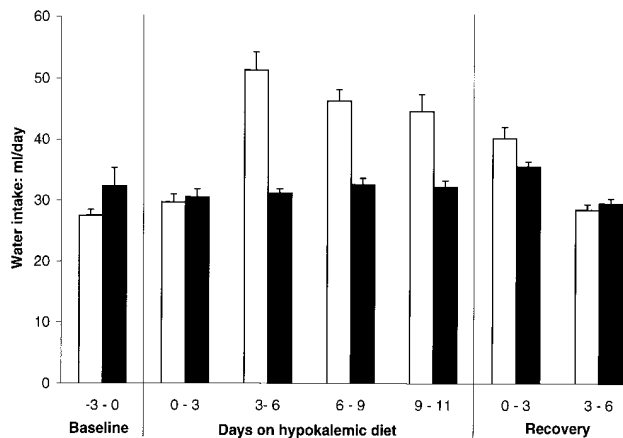


Figure 7. Water intake in control animals (solid bars), and in animals given a potassium-deficient diet for 11 d, and then a normal diet for a further seven days (open bars). Polydipsia develops over the first week on the potassium-deficient diet, and recovers rapidly upon return to a normal diet.

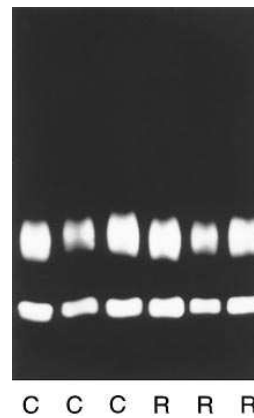


Figure 8. Immunoblot showing the effect of return to a normokalemic diet for one week. Membrane protein (5 μg) was loaded in each lane, and blots labeled with affinity-purified antibody against AQP2. There was no significant difference in AQP2 expression between controls (C) and animals recovering (R) from hypokalemia.

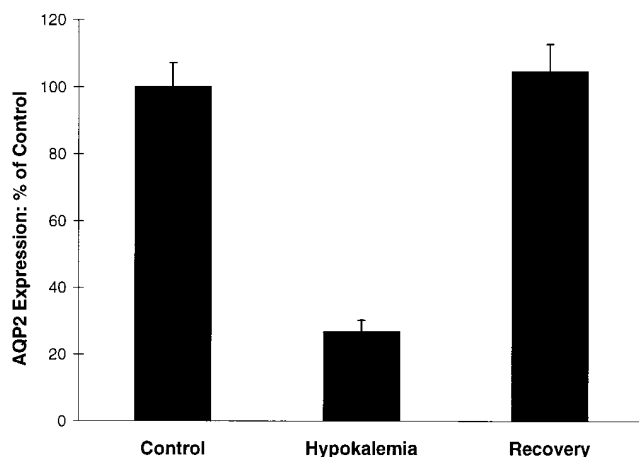


Figure 9. AQP2 expression in control animals, after 11 d hypokalemia, and after a 7-d recovery period on a normal diet. Hypokalemia causes a substantial decrease in AQP2 levels, which recover completely within one week on a normal (potassium-containing) diet.

cial cells may reflect the differences previously described in vasopressin responsiveness between cells (28, 29), and hence intracellular cAMP levels, which appear to be the signal modulating expression, as discussed above.

As previously described, vasopressin causes a redistribution of AQP2 from scattered intracellular vesicles to the apical plasma membrane (7–10). It seems likely that circulating vasopressin levels will be high in animals with a chronic polyuria, and as seen in Fig. 3, at least some cells in the collecting ducts of the hypokalemic animals appear to retain the ability to redistribute their AQP2 in response to endogenous vasopressin. This is consistent with the relatively modest effects on urine production of hypokalemia relative to those of lithium therapy (12), since lithium causes a dramatic polyuria and polydipsia, even at 10 days, when the reduction of AQP2 levels is still comparable with that seen in the present study. It is also consistent with clinical observations that hypokalemia causes a defect in maximal concentrating ability, rather than a severe polyuria (1, 17).

The granules characteristic of chronic hypokalemia (21, 22), which are believed to represent lysosomal structures (1, 23), were labeled for AQP2. Thus they may represent a site of AQP2 degradation, and hence may be involved in the decrease

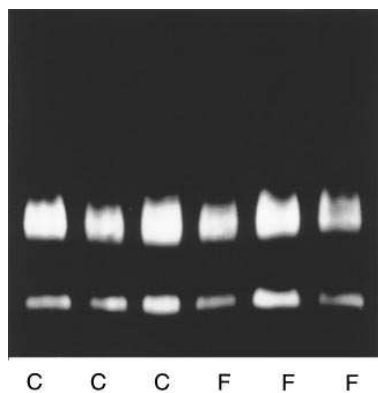


Figure 10. Immunoblot showing the effect of furosemide on AQP2 expression. Membrane protein (10 µg) was loaded in each lane, and blots labeled with affinity-purified antibody against AQP2. There was no significant difference in AQP2 expression between controls (C) and animals treated with furosemide (F) for 26 h.

in AQP2 levels observed in this study. AQP2 has previously been found in association with multi-vesicular bodies (4), and the granules, which also contain multiple small vesicular structures, may reflect a distortion of this normal cell compartment.

Summary

In summary, our results demonstrate that hypokalemia results in a decrease in AQP2 expression in both cortex and inner medulla of rat kidney, in parallel with the development of polyuria. Our results demonstrate that changes in interstitial tonicity are not a major factor in the decreased AQP2 expression, and are consistent with the hypothesis that there is long-term modulation of AQP2 expression by vasopressin, mediated by V_2 receptors and possibly cAMP. In conjunction with our findings of reduced AQP2 expression after lithium treatment (12) and bilateral ureteric obstruction (24), these results strongly suggest that a decrease in AQP2 expression may be a common feature of many polyuric states, and may contribute to the slow recovery from many such clinical conditions (30).

Acknowledgments

The authors thank Trine Møller, Mette Vistisen, Annette Pedersen, Hanne Weiling, Inger Kristoffersen, and Hanne Sidelmann for excellent technical assistance. Support for this study was provided by the Novo Nordisk Foundation, The Danish Research Academy, The Danish Medical Research Council, University of Aarhus Research Foundation, Biomembrane Research Centre, the Danish Foundation for the Advancement of Medical Science, and the intramural budget of the National Heart, Lung, and Blood Institute of the NIH.

References

- Mujais, S.K., and A.I. Katz. 1992. Potassium deficiency. In *The Kidney: Physiology and Pathophysiology*. D.W. Seldin and G. Giebisch, editors. Raven Press Ltd., New York. 2249–2278.
- Beck, N., and S.K. Webster. 1976. Impaired urinary concentrating ability and cyclic AMP in K⁺-depleted rat kidney. *Am J. Physiol.* 231:1204–1208.
- Fushimi, K., S. Uchida, Y. Hara, Y. Hirata, F. Marumo, and S. Sasaki. 1993. Cloning and expression of apical membrane water channel of rat kidney collecting tubule. *Nature (Lond.)*. 361:549–552.
- Nielsen, S., S.R. DiGiovanni, E.I. Christensen, M.A. Knepper, and H.W. Harris. 1993. Cellular and subcellular immunolocalization of vasopressin-regulated water channel in rat kidney. *Proc. Natl. Acad. Sci. USA*. 90:11663–11667.
- DiGiovanni, S.R., S. Nielsen, E.I. Christensen, and M.A. Knepper. 1994. Regulation of collecting duct water channel expression by vasopressin in Brattleboro rat. *Proc. Natl. Acad. Sci. USA*. 91:8984–8988.
- Deen, P.M., M.A. Verdijk, N.V. Knoers, B. Wieringa, L.A. Monnens, C.H. van-Os, and B.A. van-Oost. 1994. Requirement of human renal water channel aquaporin-2 for vasopressin-dependent concentration of urine. *Science (Wash. DC)*. 264:92–95.
- Nielsen, S., C.L. Chou, D. Marples, E.I. Christensen, B.K. Kishore, and M.A. Knepper. 1995. Vasopressin increases water permeability of kidney collecting duct by inducing translocation of aquaporin-CD water channels to plasma membrane. *Proc. Natl. Acad. Sci. USA*. 92:1013–1017.
- Marples, D., M.A. Knepper, E.I. Christensen, and S. Nielsen. 1995. Redistribution of Aquaporin-2 water channels induced by vasopressin in rat kidney inner medullary collecting duct. *Am. J. Physiol.* 269:C655–C664.
- Sabolic, I., T. Katsura, J.M. Verbavatz, and D. Brown. 1995. The AQP2 water channel: effect of vasopressin treatment, microtubule disruption, and distribution in neonatal rats. *J. Membr. Biol.* 143:165–175.
- Yamamoto, T., S. Sasaki, K. Fushimi, K. Ishibashi, E. Yaoita, K. Kawasaki, F. Marumo, and I. Kihara. 1995. Vasopressin increases AQP-CD water channel in apical membrane of collecting duct cells in Brattleboro rats. *Am. J. Physiol.* 268:C1546–C1551.
- Hayashi, M., S. Sasaki, H. Tsuganezawa, T. Monkawa, W. Kitajima, K. Konishi, K. Fushimi, F. Marumo, and T. Saruta. 1994. Expression and distribution of aquaporin of collecting duct are regulated by vasopressin V_2 receptor in rat kidney. *J. Clin. Invest.* 94:1778–1783.
- Marples, D., S. Christensen, E.I. Christensen, P.D. Ottosen, and S. Nielsen. 1995. Lithium-induced downregulation of aquaporin-2 water channel expression in rat kidney medulla. *J. Clin. Invest.* 95:1838–1845.

13. Gullner, H.G., A.K. Graf, J.R. Gill, and M.D. Mitchell. 1983. Hypokalaemia stimulates prostacyclin synthesis in the rat. *Clin. Sci.* 65:43-46.
14. Raymond, K.H., M.D. Lifschitz, and T.D. McKinney. 1987. Prostaglandins and the urinary concentrating defect in potassium-depleted rabbits. *Am. J. Physiol.* 253:F1113-F1119.
15. Galvez, O.G., B.W. Roberts, W.H. Bay, and T.F. Ferris. 1976. Studies of the mechanism of polyuria with hypokalemia. *Kidney Int.* 10:583a (Abstr.)
16. Berl, T., G.A. Aisenbrey, and S.L. Linas. 1980. Renal concentrating defect in the hypokalemic rat is prostaglandin independent. *Am. J. Physiol.* 238:F37-F41.
17. Carney, S., B. Rayson, and T. Morgan. 1976. A study in vitro of the concentrating defect associated with hypokalaemia and hypercalcaemia. *Pflugers Arch.* 366:11-17.
18. Gutsche, H.U., L.N. Peterson, and D.Z. Levine. 1984. In vivo evidence of impaired solute transport by the thick ascending limb in potassium-depleted rats. *J. Clin. Invest.* 73:908-916.
19. Elger, M., L. Bankir, and W. Kriz. 1992. Morphometric analysis of kidney hypertrophy in rats after chronic potassium depletion. *Am. J. Physiol.* 262:F656-F667.
20. Nielsen, S., B. Smith, E.I. Christensen, M.A. Knepper, and P. Agre. 1993. CHIP28 water channels are localized in constitutively water-permeable segments of the nephron. *J. Cell Biol.* 120:371-383.
21. Sarkar, K., and D.Z. Levine. 1975. A correlated study of kidney function and ultrastructure in potassium-depleted rats. *Nephron.* 14:347-360.
22. Sarkar, K., and D.Z. Levine. 1979. Ultrastructural changes in the renal papillary cells of rats during maintenance and repair of profound potassium depletion. *Br. J. Exp. Pathol.* 60:120-129.
23. Aithal, H.N., F.G. Toback, S. Dube, G.S. Getz, and B.H. Spargo. 1977. Formation of renal medullary lysosomes during potassium depletion nephropathy. *Lab. Invest.* 36:107-113.
24. Frøkiær, J., D. Marples, M.A. Knepper, and S. Nielsen. 1995. Bilateral ureteral obstruction downregulates expression of the vasopressin sensitive aquaporin-2 water channel in rat kidney. *Am. J. Physiol.* In press.
25. Raymond, K.H., K.K. Davidson, and T.D. McKinney. 1985. In vivo and in vitro studies of urinary concentrating ability in potassium-depleted rabbits. *J. Clin. Invest.* 76:561-566.
26. Christensen, S., E. Kusano, A.N. Yusufi, N. Murayama, and T.P. Dousa. 1985. Pathogenesis of nephrogenic diabetes insipidus due to chronic administration of lithium in rats. *J. Clin. Invest.* 75:1869-1879.
27. Christensen, S. 1988. Vasopressin and renal concentrating ability. In *Lithium Therapy Monographs*, vol. 2. F.N. Johnson, editor. Karger, S., Basel. 20-34.
28. Kachadorian, W.A., J. Muller, and V.A. DiScala. 1989. Variability of cellular responsiveness to ADH stimulation in toad urinary bladder. *Am. J. Physiol.* 256:F590-F595.
29. Nielsen, S., J. Muller, and M.A. Knepper. 1993. Vasopressin- and cAMP-induced changes in ultrastructure of isolated perfused inner medullary collecting ducts. *Am. J. Physiol.* 265:F225-F238.
30. Epstein, F.H., C.R. Kleeman, and A. Hendrikx. 1957. The influence of bodily hydration on the renal concentrating process. *J. Clin. Invest.* 36:629-634.