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Mechanism of antidiuresis caused by bendroflumethiazide in conscious rats with diabetes insipidus

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1 The mechanism underlying the antidiuretic effect of thiazide diuretics in diabetes insipidus (DI) is unknown. This study addressed two specific questions: is the reduction in urine flow rate (V) related to a decrease in the delivery of fluid from the pars recta of the proximal tubules ('distal delivery'), and are there any changes in the expression and/or intracellular distribution of vasopressin stimulated water channels (AQP2) in the collecting ducts, during chronic thiazide-induced antidiuresis?

2 Nine Brattleboro rats with vasopressin-deficient DI were treated for 5 days with bendroflumethiazide (BFTZ), 9 mg kg^{-1} day^{-1} orally, and 9 Brattleboro rats were left untreated. BFTZ-treated DI rats showed a fall in V from $\sim$200 to $\sim$75 ml day^{-1} and an increase in urine osmolality from $\sim$130 to $\sim$400 mosmol kg^{-1}.

3 BFTZ-induced antidiuresis was associated with a persistent loss of sodium, but not of potassium. After 5 days of treatment, clearance studies in conscious rats showed a tendency towards decreases in effective renal plasma flow (−7%), GFR (−12%) and lithium clearance (C_{Li}; used as marker for distal delivery) (−25%), compared with untreated controls, but none of these changes were statistically significant. There was no apparent relationship between C_{Li} and V in BFTZ-treated or untreated DI rats.

4 BFTZ treatment did not change the expression of AQP2 in homogenates of cortex, outer or inner medulla from DI rats, or from normal Long Evans rats. Light and electron microscopic immunocytochemistry revealed no changes in intracellular distribution of AQP2 in principal cells from inner medullary collecting ducts of BFTZ-treated DI rats.

5 We concluded, (i) that although the antidiuretic effect of BFTZ in rats with DI is associated with a net loss of Na, the decrease in V shows no association with changes in distal delivery, as estimated by C_{Li}. (ii) Antidiuretic treatment with BFTZ does not alter the expression of subcellular distribution of AQP2 water channels in the collecting ducts. The mechanism underlying the chronic antidiuresis caused by thiazide diuretics in DI remains elusive.

Keywords: Bendroflumethiazide; thiazide diuretics; antidiuresis; diabetes insipidus; lithium clearance; vasopressin; AQP2 water channels

Introduction

In 1959, Crawford and Kennedy showed that treatment with chlorothiazide could lower the urine flow and increase the urine osmolality both in rats and patients with diabetes insipidus (DI) (Crawford & Kennedy, 1959). The mechanism of this 'paradoxical' antidiuretic effect was, according to the authors, obscure and despite numerous investigations during the past 35 years we are still unable to explain exactly how thiazide diuretics act as antidiuretics. However, the lack of knowledge has not been an obstacle to the application in clinical medicine, where these drugs have proved valuable in the management of DI, particularly in the nephrogenic forms of this syndrome (Hays, 1992; Ives & Warnock, 1995). In DI, water permeability across the collecting duct epithelium is limited due to absence of antidiuretic hormone (ADH) or impaired response to this hormone. Consequently, water cannot be effectively abstracted when the hypotonic pre-urine passes through the collecting duct system, resulting in polyuria. Under these conditions, distal water reabsorption and hence urinary concentration ability depends on the three key factors: (i) the flow rate of tubular fluid delivered to the collecting duct system, (ii) the medullary interstitial osmolality and (iii) the constitutive (ADH-independent) water permeability across the collecting duct epithelium. And in fact, each of these factors has been implicated in the past to explain the 'thiazide paradox'.

(i) Acute thiazide administration may reduce the glomerular filtration rate (GFR) and hence distal delivery of tubular fluid (Harvard & Wood, 1962; Skadhauge, 1963; Weinman & Eknoyan, 1975; Shirley *et al*., 1978) but in several studies antidiuresis was observed without measurable changes in GFR (Earley & Orloff, 1962; Robson & Lambie, 1962; Dies *et al*., 1963; Shirley *et al*., 1982). During chronic thiazide treatment, a reduction in distal delivery may be caused by enhancement of fluid reabsorption in the proximal tubules (Weinman & Eknoyan, 1975; Walter & Shirley, 1986; Lunau *et al*., 1994). However, in the only study with simultaneous recording of distal delivery and urine flow during chronic thiazide antidiuresis, the allover change in distal delivery (−15%) was considered to be too small to account for the profound antidiuresis (Shirley *et al*., 1982). So far, no study has examined the relationship between changes in DD and urine flow during thiazide antidiuresis.

(ii) The cortico-papillary osmotic gradient would not be expected to be changed by thiazide diuretics acting on the cortical distal tubules. Accordingly, neither Baer *et al*. (1962) nor Walter & Shirley (1986) found any change in papillary osmolality in normal rats treated with thiazides. Steven & Skadhauge (1969) observed a decrease in urinary concentrating ability of normal rats given bendroflumethiazide. However, in order to contribute to the antidiuresis, medullary tonicity

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should be increased by thiazide treatment and it is difficult to imagine a mechanism for such an effect. Nevertheless, Shirley *et al.* (1982) showed that the antidiuresis induced in Brattleboro rats by chronic hydrochlorothiazide (HCTZ) administration was associated with a significant rise in papillary osmolality (from 451 to 692 mosmol kg^{-1}), an observation which led the authors to suggest that an increase in osmotic driving forces could mediate the antidiuresis. However, it remains to be demonstrated whether a possible increase in papillary interstitial osmolality in thiazide-treated DI rats is the cause or the consequence of the antidiuresis. It is known that the cortico-medullary osmotic gradient is markedly influenced by the urine flow rate *per se* and can be dissipated during water diuresis (Hai & Thomas, 1969).

(iii) The possibility that thiazide diuretics can directly affect water permeability in the collecting duct epithelium, by some ADH-like action, was suggested by Calesnick & Brenner (1961), but until now this hypothesis has only been tested experimentally in a non-mammalian epithelium, the toad isolated bladder (Pendleton *et al.*, 1968). In that preparation bendroflumethiazide (BFTZ) did not affect water permeability, nor did it potentiate the hydroosomotic effect of ADH.

The primary aim of this study in conscious rats was to assess the role of changes in the output of tubular fluid from the proximal tubules for the antidiuretic effect of thiazides in DI. The widely accepted hypothesis (Hays, 1992; Ives & Warnock, 1995) suggests that thiazide-induced sodium depletion causes a reduction in distal delivery associated with enhanced fractional water reabsorption in the collecting duct system. If this were true, there should not only be a significant decrease in distal delivery during thiazide antidiuresis, but in addition thiazide-induced changes in DD and urine flow should display some — not necessarily linear — relationship. This was tested by use of lithium clearance as a marker for distal delivery in Brattleboro DI rats treated chronically with bendroflumethiazide (BFTZ). In addition, we used immunoblotting and immuno-cytochemistry to look for BFTZ-induced changes in expression or subcellular distribution of vasopressin-sensitive water channels (AQP2) in the collecting ducts. To examine a possible interaction between ADH and BFTZ on renal haemodynamics, tubular function and AQP2 expression, a parallel series of experiments was performed in normal Long Evans rats. The study was performed in conscious and chronically instrumented rats, a model where the kidney function is uninfluenced by acute surgical stress and anaesthesia, factors which might have influenced the results of previous micropuncture studies (Weinman & Eknoyan 1975; Shirley *et al.*, 1978; 1982).

Methods

Twenty female homozygous Brattleboro rats with hereditary DI were purchased from Harlan Sprague Dawley Inc. (Zeist, Holland) and 20 female Long Evans (LE) rats were purchased from Møllegaard Breeding Centre (L1. Skensved, Denmark). The body weight was about 200 g at the delivery and rats were housed under controlled conditions (temperature 22–24°C; relative humidity 40–60%; lights on from 08 h 00 min to 20 h 00 min) at the Panum Institute Department of Experimental Medicine. A balance study with quantitative assessment of food intake and electrolyte excretions was performed for 12 days, by use of Techniplast model 1700 metabolism cages (Scanbur A/S, Lellinge, Denmark). Throughout the balance study, rats received distilled water *ad libitum* and a granulated diet (Altromin #1310; Altromin International, Lage, Germany) which contained: Na 140 mmol kg^{-1}, K 275 mmol kg^{-1}, Li 7 or 12 mmol kg^{-1} and proteins 190 g kg^{-1}. Lithium citrate was added to the diet in order to produce a pharmacologically inactive but measurable plasma concentration of this ion (0.15–0.20 mmol l^{-1}), the lower dose being used for rats treated with BFTZ (Leo Pharmaceuticals, Ballerup, Denmark). The latter compound was added in amounts of 125 mg kg^{-1} diet.

Experimental groups and time schedule

The animals were placed randomly into four experimental groups, with 9–10 rats in each: group 1: DI controls; group 2: DI + BFTZ; group 3: LE controls; group 4: LE + BFTZ. On day 0 a Tygon medical grade catheter was implanted in the femoral vein under brief anaesthesia (Petersen *et al.*, 1991). At 8 h 00 min two days later, after recovery from the operation, the rats were placed in individual metabolic cages with access to distilled water and granulated lithium-containing diet. Body weight, water intake and consumption of diet were recorded daily. Twenty-four-hour balances of Na and K were calculated as electrolyte intake minus the amount excreted in the urine. After 3 baseline 24 h collections, the control diet was replaced with BFTZ-containing diet in groups 2 and 4, and on day 10 renal functional variables were assessed in all groups by clearance technique. Finally, on day 12 the animals were anaesthetized and the kidneys were prepared for determination of Aquaporin-2 (AQP2) expression and subcellular distribution.

Clearance protocol

The clearance experiments on day 10 were performed in the metabolic cages with free access to water and diet. At 8 h 00 min, i.v. infusion of 150 mM glucose was started with a rate that approximated the 24 h urine flow rate, i.e. 8 ml h^{-1} in group 1, 4 ml h^{-1} in group 2 and 2 ml h^{-1} in groups 3–4. [^{3}H]-inulin (Amersham, Buckinghamshire, U.K.; batch no. 135; specific activity 3.1 Ci $mmol^{-1}$) and [^{14}C]-tetraethylammonium bromide (NEN, Boston, MA, U.S.A.; lot no. 2967-044; specific activity 5.0 mCi $mmol^{-1}$) were added to the infusate in amounts of 5 and 1.66 μCi h^{-1}, respectively, as markers for the glomerular filtration rate (GFR) and the effective renal plasma flow (ERPF). A small amount of LiCl (6 μmol h^{-1}) was added to maintain plasma lithium levels during the clearance study. After a 90 min equilibration period, 250 μl of blood was sampled from the tail tip and the rats were provoked to empty their bladders. The urine was collected for 1 h in DI rats and for 2 h in LE rats and a second blood sample was obtained. Rats were then given an i.v. bolus of 0.25 mg amiloride hydrochloride (MSD, Harlem, The Netherlands) followed by a constant infusion of 0.5 mg h^{-1}. A second 1 or 2 h urine collection was commenced 30 min after the start of the amiloride infusion, and the experiment was terminated by a third blood sample. To facilitate bladder emptying, rats were placed for 1–2 min on a smooth glass surface and the bladder was compressed by applying a light suprapubic pressure.

Analytical methods

Electrolytes in diet, plasma and urine were determined after appropriate extractions and dilutions, by use of a Perkin Elmer model 2380 atomic absorption spectrometer (Perkin Elmer, Allerød, Denmark). For determination of ^{3}H-inulin and ^{14}C-tetraethylammonium, 15 μl samples were mixed with 85 μl distilled water and 2.5 ml Ultima Gold (Packard Instruments,

Greve, Denmark) and counted in a Packard TriCarb liquid scintillation analyser model 2250CA, by use of a dual label DPM programme and transformed spectral index of external standard as quench indicating parameter. Urine osmolalities were determined in an Advanced cryomatic osmometer, model 3CII (Advanced Instruments, Needham Heights, Massachusetts, U.S.A.), with sodium chloride solutions as standards.

Kidney preparation and expression and subcellular distribution of AQP2 water channels

Rats were anaesthetized with halothane + N_20 and the right kidney was ligated, removed and frozen in liquid nitrogen. The left kidney was perfused *in situ* for 3 min with 4% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.2, after which it was removed and postfixed in cacodylate buffer with 2% paraformaldehyde. Blocks were cut from cortex, outer medulla, and inner medulla, and infiltrated with 2 M sucrose solution before being frozen in liquid nitrogen. For determination of APQ2 expression in each renal zone, crude membrane fractions were prepared from cortex, outer medulla and inner medulla of the right kidney, and AQP2 was quantitated by Western blotting, with a peptide-derived antibody raised against the COOH-terminal 22 amino acids of AQP2. Ten micrograms of membrane protein were loaded in each lane and quantification of ECL exposures of immunoblots was performed by densitometry including all bands (non-glycosylated, glycosylated and oligomeric AQP2) (Marples *et al*., 1995). For determination of subcellular distribution of AQP2 in inner medullary collecting ducts, light microscopic immunocytochemistry was performed on 0.85 μm cryosections of the left kidney, as previously described (Marples *et al*., 1995). In addition, immunoelectron microscopy was performed: Tissue blocks from the left kidney were subjected to freeze-substitution and embedded in Lowicryl HM-20 by use of procedures previously described (Nielsen *et al*., 1995). The frozen samples were freeze-substituted in a Reichert AFS (Reicher, Vienna, Austria). Samples were sequentially equilibrated over 3 days in 0.5% uranyl acetate in methanol at temperatures gradually increasing from -80°C to -70°C, and then rinsed in pure methanol for 14 h at -70°C to -45°C and infiltrated at -45°C with Lowicryl HM-20 and methanol 1:1, 2:1 and finally pure Lowicryl HM-20 (one day in each solution) before u.v. polymerization in pure HM-20 for 2 days at -45°C and 2 days at 0°C. Immunolabelling was performed on 0.05 μm sections incubated with affinity-purified aquaporin-2 antibody and visualized with goat-anti-rabbit IgG conjugated to 10 nM colloidal gold particles (GAR.EM10; BioCell Research Laboratories, Cardiff, U.K.). Sections were stained for 5 s with lead citrate and for 10 min with uranyl acetate and examined in a Philips CM100 electron microscope at a primary magnification of 28,000×. The following controls confirmed specificity of light and electron microscopic immunolabellings: (i) incubation with non-immune rabbit IgG; (ii) incubation without primary antibody or secondary antibody.

Calculations and statistics

Renal clearances (C) and fractional excretions (FE) were calculated by the standard formula:

$$C = U \times V/P;\ FE = C/GFR$$

where U = urine concentration, P = plasma concentration and V = urine flow rate. C_{In} was used as a marker for GFR and C_{TEA} as a marker for effective renal plasma flow (EFRF) (Petersen *et al*., 1991). C_{Li} was used as an indirect marker for distal delivery, defined in this paper as the flow of tubular fluid leaving the pars recta of the proximal tubule (Thomsen & Shirley, 1997). Free water clearance was calculated from the formula $C_{H20} = V$ (1-U_{osm}/295). All values are presented as mean ± s.e.mean. Paired or unpaired Student's t-test was used, where appropriate, to evaluate differences between periods of groups. The level of significance was set to $P < 0.05$.

Results

Balance study in DI rats

While housed in metabolic cages, untreated DI rats maintained a constant weight, whereas those treated with BFTZ lost about 6 g or 3% during the first days of treatment (Figure 1a). BFTZ treatment did not alter the average daily food intake (14.9 ± 0.1 g in group 2 vs 15.7 ± 0.2 g in group 1; NS). The mean calculated dose of BFTZ was 1.86 mg daily or 9.3 mg kg^{-1} day^{-1}. Untreated DI rats showed a daily urine production similar to their own body weight, and a urine osmolality of about 130 mosmol kg^{-1} (Figure 1b and 1c). Treatment with BFTZ reduced the urine flow to about 75 ml day^{-1} and increased the urine osmolality towards 400 mosmol kg^{-1}. Free water clearance (Figure 1d) was reduced from about 115 ml day^{-1} to negative values during BFTZ treatment. In the control group, for unknown reasons there was a spontaneous decline in free water clearance to 65 ml day^{-1} during the observation period. However, the changes induced by BFTZ were highly significant at any time.

Untreated DI rats showed a positive balance for sodium and potassium (Figure 1e and 1f) which probably reflects (undetermined) faecal electrolyte losses. Administration of BFTZ caused an initial sodium loss of about 1 mmol on the first day of treatment, and this deficit was maintained during the rest of the study. BFTZ did not cause any significant loss of potassium in DI rats.

Balance study in LE rats

In normal (LE) rats BFTZ caused a small weight loss and a persistent loss of both sodium and potassium. No significant changes were observed in urine flow, urine osmolality or free water clearance (Figure 2). In LE rats, the mean calculated dose of BFTZ was 1.65 mg day^{-1} or 7.8 mg kg^{-1} day^{-1}.

Renal variables on day 10

The results of the clearance study on day 10 (before administration of amiloride) are shown in Table 1. The antidiuresis induced by 5 days BFTZ treatment in DI rats was associated with a tendency towards a lowering of ERPF (-7%), GFR (-12%), C_{Li} (-25%) and FE_{Li} (-16%) when compared to untreated controls. However, none of these changes was statistically significant. In LE rats, chronic administration of BFTZ caused no changes in V, but significant decreases in ERPF (-18%), C_{Li} (-30%) and FE_{Li} (-25%).

Acute amiloride administration, used in this study to check for possible distal lithium reabsorption induced by thiazide treatment (Shalmi & Thomsen; 1993), did not enhance C_{Li} in either group (Table 2). Amiloride caused a small but statistically significant increase in FE_{Li} in untreated DI and

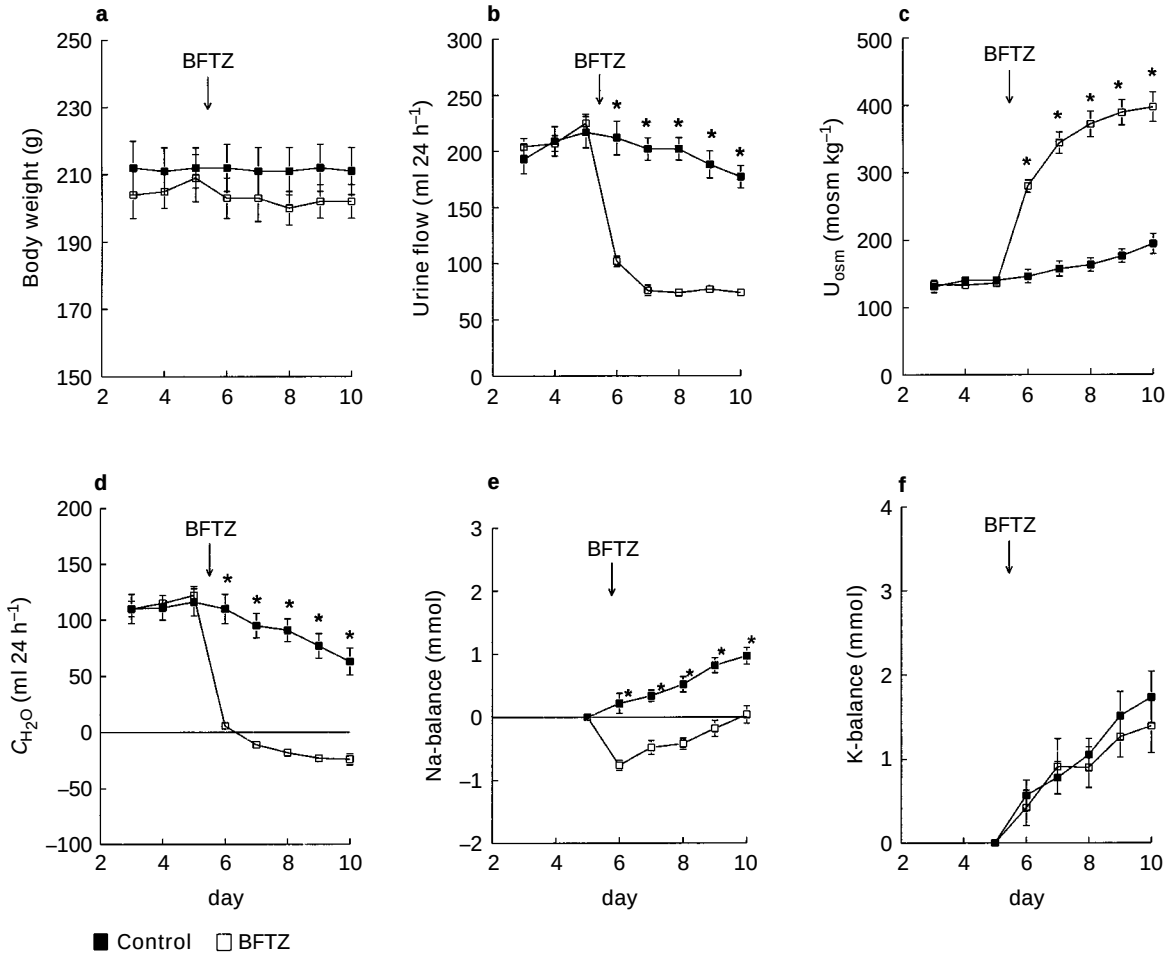

Figure 1 Body weight (a), urinary concentration variables (b, c and d) and electrolyte balances (e and f) in Brattleboro DI rats before and during treatment with bendroflumethiazide (BFTZ). BFTZ was mixed with the diet (125 mg kg^{-1} ~9 mg kg^{-1} b.w. day^{-1}) on day 6 as indicated by arrow. Mean values are shown; vertical lines indicate s.e.mean. Asterisks indicate significant differences ($P<0.05$) vs control group.

Table 1 Renal variables in the four experimental groups after 5 days treatment with bendroflumethiazide (BFTZ; 8–9 mg kg^{-1} b.w. daily)

	Group 1 DI controls (n=7)	*Group 2 DI+BFTZ (n=9)*	*Group 3 LE controls (n=9)*	*Group 4 L+BFTZ (n=9)*
V (μl min^{-1} 100 g^{-1} bw)	57±7	31±4*	14±2	16±2
C_{TEA}~ERPF (μl min^{-1} 100 g^{-1} bw)	3,148±238	2,917±145	4,513±198	3,719±133*
C_{In}~GFR (μl min^{-1} 100 g^{-1} bw)	837±46	736±34	1,060±38	992±29
C_{Li} (μl min^{-1} 100 g^{-1} bw)	251±27	189±24	363±30	255±19*
FE_{Li} (%)	30±3	26±3	34±2	26±2*

Mean values and s.e.mean are indicated. Data are based on 1 h urine collection periods for DI rats and 2 h collection periods for LE rats. *Denotes statistical differences vs the corresponding control group ($P<0.05$). V, urine flow rate; ERPF, effective renal plasma flow; GFR, glomerular filtration rate; C, renal clearance; FE, fractional excretion.

Table 2 Effects of i.v. amiloride administration (0.25 mg bolus; 0.5 mg h^{-1}) on renal lithium clearance (C_{Li}) and fractional lithium excretion (FE_{Li}) in the four experimental groups

	C_{Li} (μl min^{-1} 100 g^{-1} bw) *Control period*	C_{Li} (μl min^{-1} 100 g^{-1} bw) *Amiloride period*	FE_{Li} (%) *Control period*	FE_{Li} (%) *Amiloride period*
DI control ($n=7$)	251±27	263±34	30.3±3.1	35.3±4.2*
DI+BFTZ ($n=9$)	189±24	201±28	25.6±3.5	30.7±4.4
LE control ($n=9$)	363±30	359±14	34.1±2.6	41.5±2.1*
LE+BFTZ ($n=9$)	255±19	168±8*	25.6±2.1	20.4±1.4*

Mean values and s.e.mean are indicated. *Denotes statistical differences in comparison with the control period (paired t test $P<0.05$).

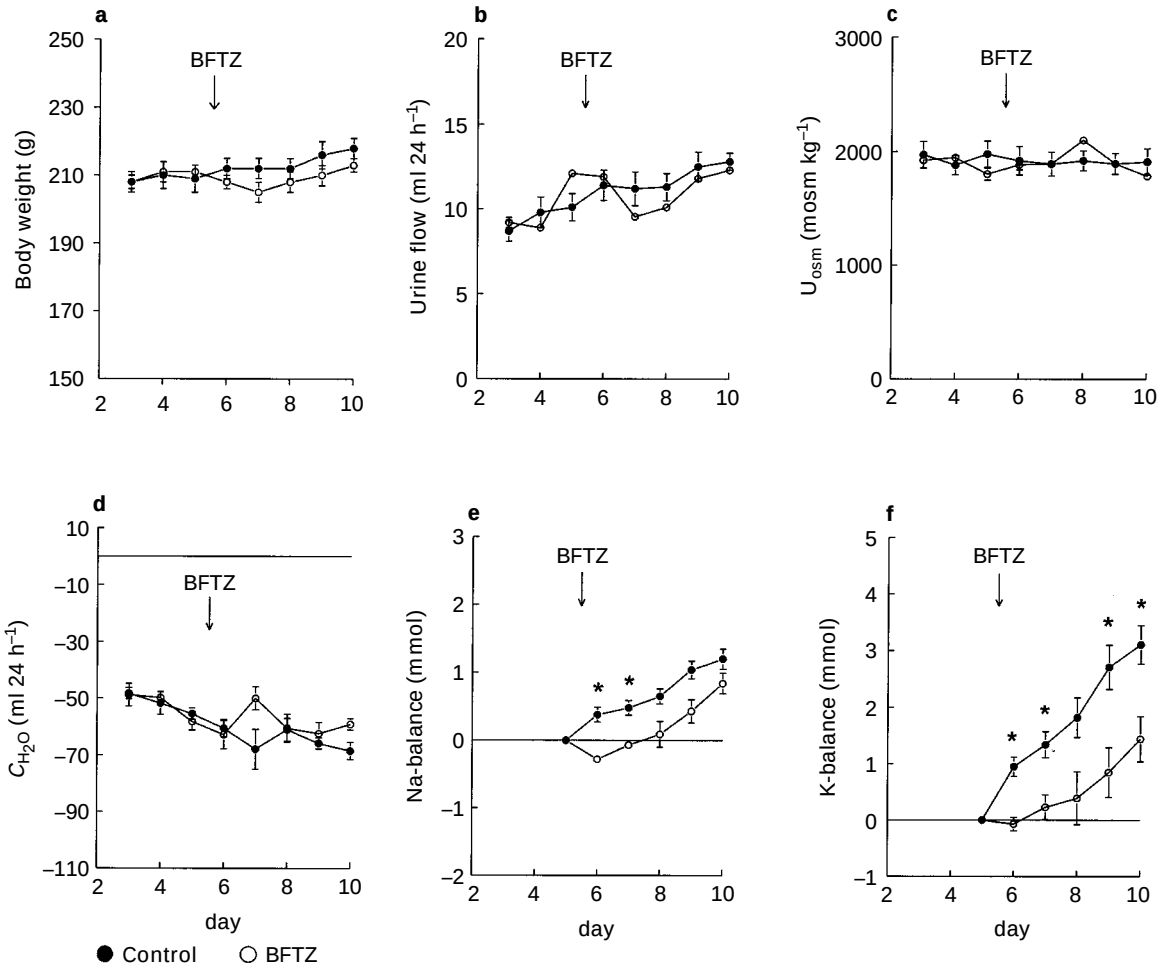

Figure 2 Body weight (a), urinary concentration variables (b, c and d) and electrolyte balances (e and f) in normal Long Evans rats before and during treatment with BFTZ. BFTZ was mixed with diet (125 mg kg^{-1} ~8 mg kg^{-1} b.w. day^{-1}) on day 6 as indicated by arrow. Mean values are shown; vertical lines indicate s.e.mean. Asterisks indicate significant differences ($P<0.05$) vs control group.

LE rats, but not in DI rats treated with BFTZ. In thiazide-treated LE rats, amiloride caused a decrease of C_{Li} and FE_{Li}. Thus, there was no indication that BFTZ induced amiloride-sensitive lithium reabsorption in either strain of rats.

Figure 3 indicates the relationship between C_{Li} (based on 1 h urine collections) and V in BFTZ-treated as well as untreated DI rats. The urine flow rate was either calculated from the 24 h urine collection before the clearance study (Figure 3a) or from the simultaneous 1 h urine collection (Figure 3b). There was no significant relationship between C_{Li} and V in any of the cases.

Expression and subcellular distribution of AQP2 water channels in the kidneys

AQP2 expression was assessed by immunoblotting on membrane fractions from cortex, outer medulla and inner medulla (Marples *et al*., 1995). Densitometry of the combined bands (non-glycosylated, glycosylated and oligomeric) from each renal zone revealed that there were no differences between BFTZ-treated and control rats of either strain. Representative examples of blots from inner medulla are shown in Figure 4. Relative whole kidney AQP2 expression (mean of all three zones) averaged 0.93 ± 0.24 ($\pm$s.e., $n=9$) in BFTZ-treated Brattleboro rats versus 1.00 ± 0.20 ($\pm$s.e., $n=6$) in control Brattleboro rats.

To determine if BFTZ-treatment affected the subcellular localization of AQP2 in Brattleboro rats, immunocytochemistry and quantitative immunelectron microscopy were performed on the perfusion fixed kidneys. Irrespective of treatment, immunoperoxidase revealed that the labelling was almost exclusively confined to intracellular domains with little or no labelling concentrated in the plasma membrane (Figure 5). This was confirmed by immunoelectron microscopy showing labelling of intracellular vesicles and multivesicular bodies, but only very low labelling of the plasma membrane (Figure 6). Quantification of the distribution of immunogold labelling showed that both in BFTZ-treated and control rats, more than 95% of the labelling was associated with intracellular vesicles and multivesicular bodies and less than 5% was associated with the apical plasma membrane. There was no difference in the subcellular distribution of AQP2 between BFTZ-treated and control Brattleboro rats (Table 3).

Discussion

In this study, the antidiuretic effect of BFTZ was investigated in Brattleboro rats with ADH-deficient DI. BFTZ-induced antidiuresis was associated with a persistent loss of sodium but not with any significant changes in region or whole-kidney

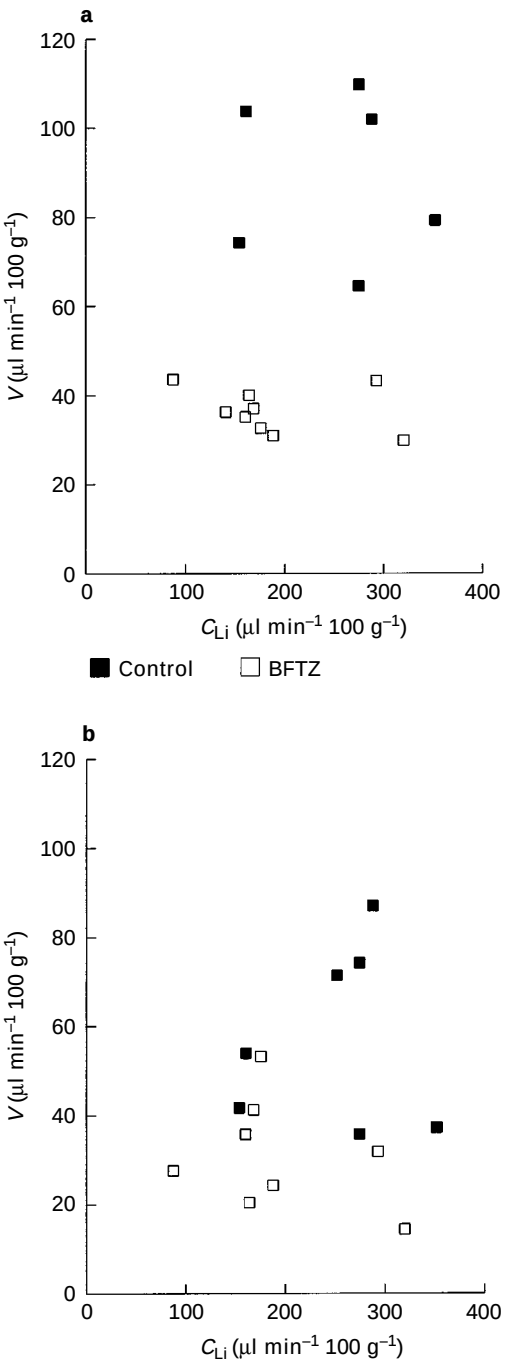

Figure 3 Relationship between lithium clearance (as indicator for distal delivery) and urine flow rate in untreated and BFTZ-treated DI rats on day 10. The lithium clearance measurement was based on 1 h urine collections. Urine flow rate was calculated from the preceding 24 h urine collection (a) or from the simultaneous 1 h urine collection (b). There was no significant correlation between the two variables in either case.

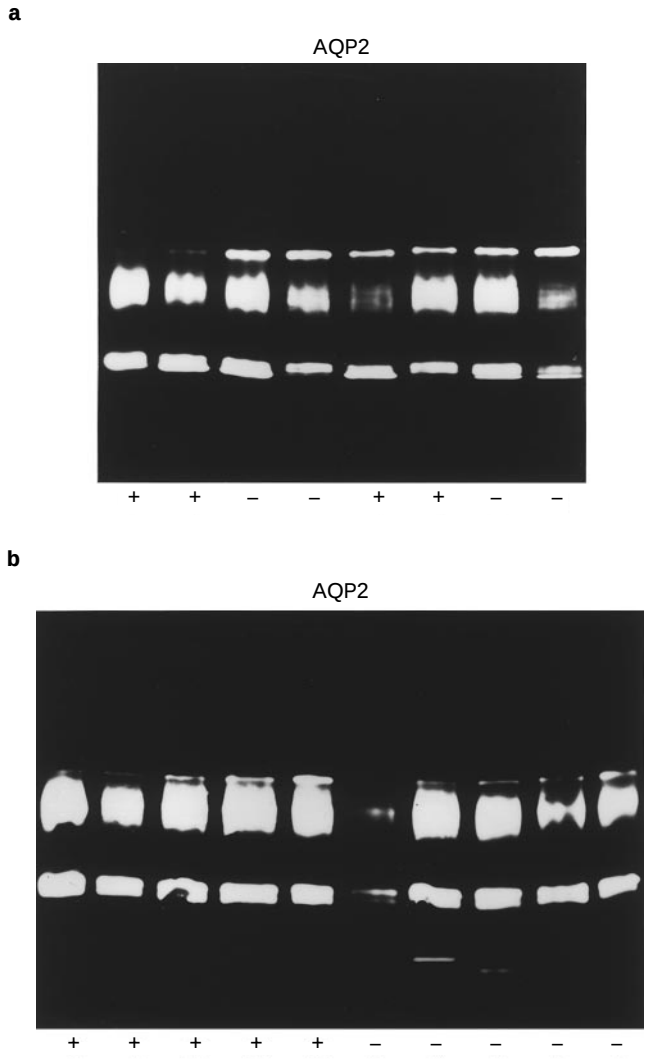

Figure 4 (a) Individual immunoblots of membrane fractions from kidney inner medulla (10 μg protein/lane) from 4 Brattleboro DI rats treated with BFTZ (+) and 4 untreated DI rats (−). The immunoblot was labelled with anti-AQP2 serum (1:1000). The lower (21 kDa) band corresponds to non-glycosylated AQP2. The medium (35–45 kDa) band represents glycosylated AQP2. The weak (approximately 55 kDa) top band probably represents oligomeric AQP2. There was no difference in relative expression, determined by densitometry of all three bands, between treated and untreated rats. The same pattern was observed in membrane fractions from outer medulla and cortex. (b) Similar individual immunoblots of membrane fractions from kidney inner medulla from 5 Long Evans rats treated with BFTZ (+) and 5 untreated Long Evans rats (−). Again, there was no difference in relative AQP2-expression between treated and untreated rats, and a similar pattern was observed in membrane fractions from outer medulla and cortex. The exposure time was less than for the DI-rats shown in (a).

APQ2 expression, or in subcellular distribution of AQP2 in the principal cells of the inner dedullary collecting ducts. More surprisingly, despite a persistent and highly significant antidiuretic response to BFTZ ($V = 74 \pm 4$ ml 24 h^{-1} in BFTZ-treated rats vs 178 ± 11 ml 24 h^{-1} in controls), the BFTZ-induced decrease of C_{Li} (used in this study as an indicator for distal delivery of tubular fluid; Thomsen & Shirley 1997) was not statistically significant, and there was, furthermore, no relationship between V and C_{Li} in BFTZ-treated and untreated DI-rats.

These results do not support the current hypothesis (Hays, 1991; Ives & Warnock, 1995) that the antidiuretic effect of thiazide diuretics in DI is due to a reduction in the flow rate of tubular fluid delivered to the distal nephron. The validity of this interpretation (i.e. that the thiazide antidiruesis occurs independently of changes in distal delivery) of course depends on the reliability of C_{Li} as a marker for distal delivery in the present experimental setting. It is generally accepted that under sodium-replete conditions C_{Li} is a fairly good, although semiquantitative indicator for the flow rate of tubular fluid leaving the pars recta of the proximal tubule (Thomsen & Shirley, 1997). However, when rats are exposed to dietary

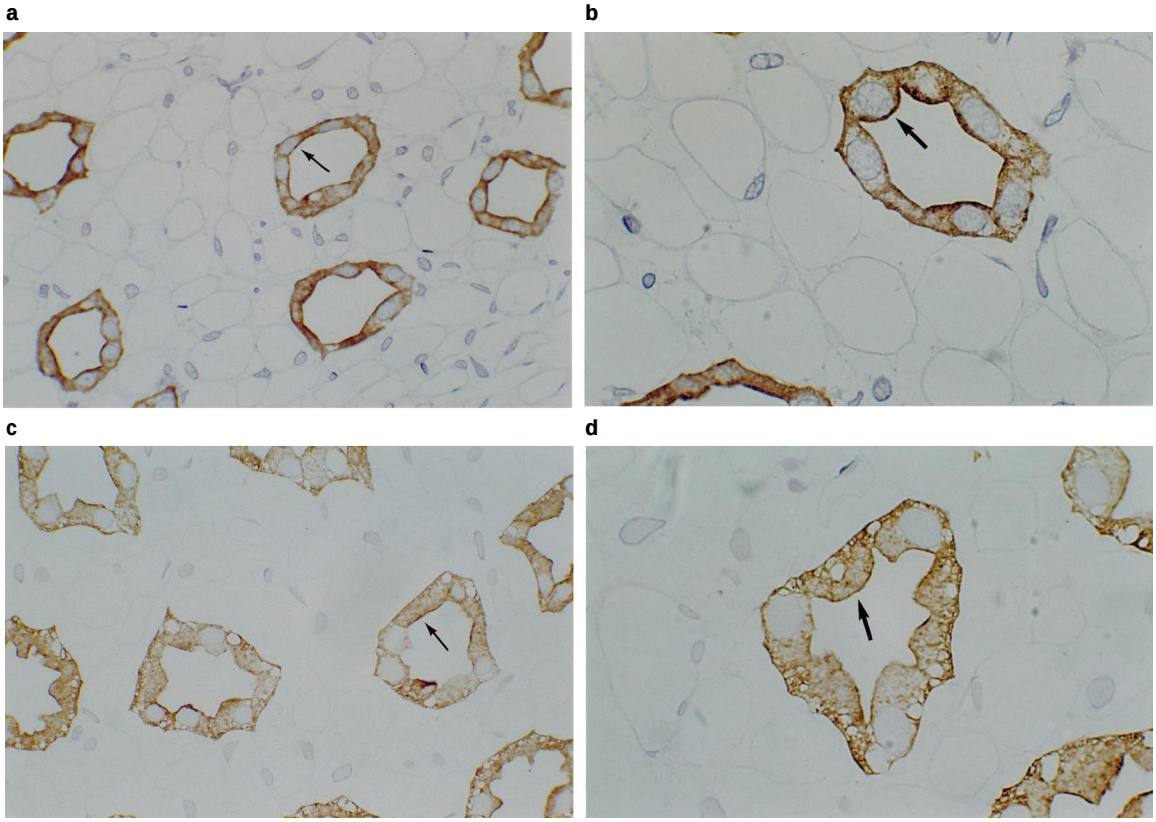

Figure 5 Light microscopic immunocytochemistry of AQP2 localization in 0.8 μm cryosections of kidney inner medulla of Brattleboro rats with diabetes insipidus. (a and b) Control rat. (c and d) BFTZ-treated rat. AQP2 labelling revealed a vesicular pattern with labelling distributed in both apical (arrows) and basal parts of the cell. Magnification: (a) and (c) $\times 480$; (b) and (d) $\times 960$.

Table 3 Subcellular distribution of AQP2 in inner medullary collecting duct cells from Brattleboro rats with vasopressin-deficient diabetes insipidus (DI).

	Fraction of labelling in intracellular vesicles and multivesicular bodies (%)	*Fraction of labelling in apical plasma membrane* (%)*	*Number of gold particles*	*Number of rats*
DI rats, BFTZ	96.2±0.7	3.8±0.7	1571	4
DI rats, Control	197.8±1.0	2.1±1.0	1049	3

*The criterion for labelling of the apical plasma membrane was a distance less than 30 nm from the outer surface. There was no difference subcellular distribution of AQP2 between BFTZ-treated and control rats.

NaCl restriction, lithium is reabsorbed in significant amounts though the amiloride-sensitive sodium channels in the collecting ducts (Shalmi & Thomsen, 1993). It may be speculated that sodium depletion caused by BFTZ could also induce distal lithium reabsorption, so that C_{Li} would not be a valid indicator during that condition. We examined this possibility by administration of a test dose of amiloride previously shown to block distal lithium reabsorption in sodium restricted rats (Shalmi & Thomsen, 1993). The observation that this manoeuvre did not cause any increase of C_{Li} or FE_{Li} in thiazide-treated rats argues against the possibility that distal lithium reabsorption was induced by BFTZ treatment.

Another possible complication in the interpretation of our data could be if spontaneous fluctuations of C_{Li} (a variable which was based on 1 h urine collections) obliterated the hypothesized relationship with urine flow. However, in that case C_{Li} should show a more distinct relationship to the 1 h urine flow than to the 24 h value, which was not observed (Figure 3). Thus, the missing relationship between 'distal delivery' and urine flow could not be attributed to any recognized confounding factor and may indeed indicate that the antidiuresis was not the result of a decreased delivery of tubular fluid to the distal nephron. On the other hand, previous studies by other investigators have demonstrated a statistically significant fall in distal delivery (Shirley *et al*., 1982; 1983) and C_{Li} (Thomsen & Schou, 1973) in Brattleboro rats treated chronically with hydrochlorothiazide. Also, it is known that prolonged thiazide treatment causes a fall in distal delivery and C_{Li} in normal rats (Thomsen & Schou, 1973; Walter & Shirley 1986; Lunau *et al*., 1994; and the present data in LE rats) as well as in man (Petersen *et al*., 1974). However, the crucial question is whether the thiazide-induced reduction in distal delivery, as demonstrated by others, can explain the marked antidiuresis occurring in subjects with DI? Shirley *et al*. (1982) found a relatively small (15%) decrease in distal delivery in thiazide-treated DI rats and concluded that this could not explain the more than 50% reduction in urine

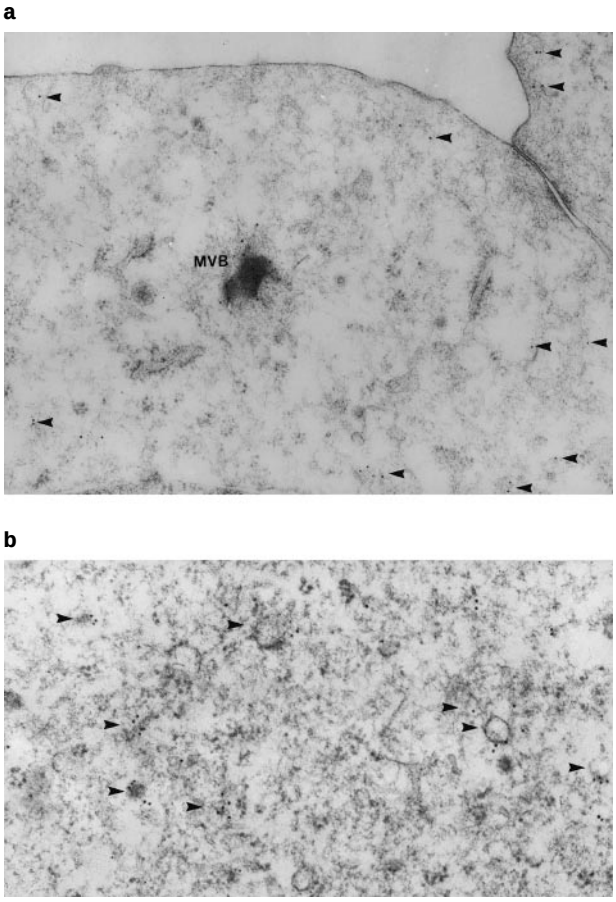

Figure 6 Immunoelectron microscopy of AQP2 labelling in an inner medullary collecting duct cell from an untreated Brattleboro rat with diabetes insipidus. (a) Apical part of the cell showing labelling of intracellular vesicles (arrows) and multivesicular body (MVB) with no labelling of the apical plasma membrane. Magnification × 84,000. (b) Central part of the cell showing AQP2 labelling confined to small intravesicular vesicles (arrowheads). Magnification × 112,000.

production; they speculated that other factors, such as increased medullary interstitial osmolality, could mediate the antidiuresis. However, they did not demonstrate a relationship between distal delivery and urine flow in thiazide-treated and untreated DI rats. Theoretically, the possibility cannot be excluded that a small decrease in distal delivery could induce a relatively larger reduction in urine flow, since fractional distal water reabsorption may be expected to increase when the tubular flow declines. However, when simultaneous values for C_{Li} and V were correlated in untreated DI rats (Shirley *et al*., 1983) or in rats with Li-induced polyuria (Thomsen, 1977) the two variables showed a strictly linear relationship, suggesting that at least in untreated DI rats distal water reabsorption is a constant fraction of the distal delivery.

To test whether BFTZ had any ADH-like action, as suggested originally by Calesnick & Brenner (1961), we looked for changes in the expression and intracellular distribution of AQP2 water channels during BFTZ treatment. Identical results were obtained from Brattleboro rats and Long Evans rats. Immunocytochemistry revealed no changes in subcellular AQP2 distribution, indicating that BFTZ does not mimic the ability of ADH to cause transfer of AQP2 from intracellular vesicles to the apical plasma membrane. Furthermore, immunoblotting revealed no change in whole-kidney or regional AQP2 expression, indicating that BFTZ was not acting by increasing AQP2 expression, as for example observed in response to dehydration (Nielsen *et al*., 1993). Consistent with the previous observation that thiazides do not affect water permeability in the toad bladder (Pendleton *et al*., 1968), our results argue strongly against the hypothesis that thiazides may exert an ADH-like effect on collecting duct water transfer.

Sodium depletion has been suggested to be a necessary stimulus for the antidiuretic effect of thiazide diuretics. Thus, Shirley *et al*. (1978) showed that the acute antidiuresis induced by HCTZ in DI rats could be prevented by sodium replacement. However, in subsequent studies the same authors demonstrated that the chronic antidiuresis elicited by HCTZ can be completely dissociated from changes in sodium balance (Walter & Shirley, 1983). In the presence study, the antidiuresis appeared to be associated with a loss of total body sodium, based on urinary sodium excretion data exclusively, since faecal Na losses were not determined. However, it seems unlikely that the minute amounts of Na normally excreted with the faeces should be influenced by BFTZ-treatment, since in a previous study in DI rats (Walter *et al*., 1982) treatment with HCTZ did not influence faecal Na content. So at the present time, despite the fact that thiazide-induced antidiuresis is almost invariably associated with Na depletion, it is not known whether a loss of sodium is a prerequisite for the antidiuresis. Recent experiments in our laboratory have shown that servo-controlled clamping of total body sodium cannot prevent the acute thiazide antidiuresis in DI rats (Spannow *et al*., 1997). In the present study, the apparent lack of relationship between changes in distal delivery and urine flow indeed argues against a sodium- or volume-dependent mechanism for thiazide-induced antidiuresis.

An interesting observation in the present study was that thiazide treatment did not induce any detectable loss of potassium in rats with DI. Since BFTZ induced a similar loss of sodium in LE and DI rats, this difference between the two strains in renal potassium handling cannot be due to a difference in the load of sodium to the Na/K exchange site in the collecting ducts. However, it is possible that DI rats, being in a permanent potassium losing state due to the high urine flow, excreted less potassium in response to thiazide administration because of the simultaneous decrease in tubular fluid flow rate at the distal site for potassium secretion.

In conclusion, our results indicate that under the experimental conditions used in this study, antidiuresis induced by BFTZ administration to rats with vasopressin-deficient DI is associated with a net loss of sodium, but dissociated from changes in distal delivery of tubular fluid, as assessed by lithium clearance. In addition, antidiuretic thiazide treatment does not change the expression or intracellular distribution of AQP2 water channels in the collecting ducts. Our results do not support current hypotheses proposed to explain the mechanism of thiazide-induced antidiuresis in diabetes insipidus.

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