Loss of calcineurin Aα results in altered trafficking of AQP2 and in nephrogenic diabetes insipidus

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Summary

The serine/threonine phosphatase calcineurin is an important signaling molecule involved in kidney development and function. One potential target of calcineurin action is the water channel aquaporin 2 (AQP2). In this study, we examined the effect of loss of calcineurin Aα (CnAα) on AQP2 function in vivo. CnAα null mice were found to have defective post-natal urine-concentrating ability and an impaired urine-concentrating response to vasopressin. Expression of AQP2 is normal but, paradoxically, vasopressin-mediated phosphorylation of the channel is decreased compared with wild-type littermates and there is no accumulation of AQP2 in the apical membrane. Calcineurin protein and activity was found in innermedullary collecting duct vesicles, and loss of calcineurin expression and activity was associated with a loss of AQP2 in the vesicle fraction. As such, the lack of vasopressin-mediated phosphorylation of AQP2 might be the result of a defect in normal trafficking of AQP2 to apical-targeted vesicles. Likewise, treatment of wild-type mice with cyclosporin A to inhibit calcineurin produces a similarly impaired urine-concentrating response to vasopressin and alterations in AQP2 phosphorylation and trafficking. These experiments demonstrate that, CnAα is required for normal intracellular trafficking of AQP2 and loss of calcineurin protein or activity disrupts AQP2 function.

Key words: Cyclosporin A, AQP2 trafficking, Calcineurin A-alpha knockout mouse

Introduction

AQP2 is the predominant vasopressin-regulated water channel of the kidney collecting duct and is essential for urinary concentration (Knepper, 1997; Nielsen et al., 1996). AQP2, a highly glycosylated protein, is processed through the endoplasmic reticulum (ER) and Golgi network where it is folded correctly and then targeted to vesicles that are directed to the subapical region of the plasma membrane. Thereafter, water permeability of the inner medullary collecting duct (IMCD) cells can be rapidly regulated by the antidiuretic hormone arginine vasopressin that binds to heptahelical vasopressin V_2 receptors (V_2R), located mainly in the basolateral membrane of principal cells (Klussmann et al., 2000; Storm et al., 2003; Ward et al., 1999). Activation of the V_2R causes stimulation of adenylyl cyclase via the G protein G_S, leading to elevation of cAMP and subsequent activation of protein kinase A (PKA) (Wade et al., 1981). PKA phosphorylates AQP2 at serine reside 256, resulting in enhanced accumulation of AQP2-bearing vesicles in the plasma membrane where AQP2 is inserted by an exocytosis-like process (Ausiello et al., 1987; Hayashi et al., 1994).

In addition to PKA, several factors participate in the normal trafficking of AQP2, including the Golgi network casein kinase (G-CK), which might phosphorylate AQP2 during processing in the Golgi (Procino et al., 2003), and A kinase anchoring proteins (AKAPs) (Klussmann et al., 1999) which are scaffolding proteins that facilitate interaction of AQP2 and PKA. Interestingly, recent data suggest that the serine/threonine phosphatase calcineurin also regulates the trafficking of AQP2. First, calcineurin binds to at least two AKAP proteins including AKAP79, which is expressed in the kidney, in close proximity to PKA (Coghlan et al., 1995). Second, Jo et al. identified calcineurin in a complex with AQP2 and an AKAP scaffold protein and showed that calcineurin dephosphorylated APQ2 in an in vitro assay (Jo et al., 2001). Our previous work also supports this idea; we found that the α isoform of calcineurin A subunit (CnAα) and AQP2 colocalize in collecting duct principal cells of normal and diabetic rats (Gooch et al., 2004a). Paradoxically, the functional consequence of calcineurin inhibition with cyclosporin A was a decrease in phosphorylation and a redistribution of AQP2 away from the apical membrane. Finally, long-term inhibition of calcineurin with cyclosporin A results in polyuria and decreased urine osmolality (Batlle et al., 1986; Lim et al., 2004) and downregulation of several aquaporins including AQP2 (Lim et al., 2004). However, it is not known whether cyclosporin A directly alters AQP2 action or whether it alters urine-concentrating capacity through other mechanisms.

The study was undertaken to further understand the role of CnAα in AQP2 trafficking in vivo. CnAα–/– mice have been created as previously reported (Zhang et al., 1996). These mice

offer a unique opportunity to evaluate the role of CnAα in AQP2 regulation and trafficking in vivo. Here, we report for the first time that CnAα null mice show decreased phosphorylation of AQP2 in response to vasopressin and significantly less AQP2 protein is found in inner medullary collecting duct vesicles and, consequently, the apical membrane. Instead, AQP2 appears to be retained in an intracellular compartment, consistent with a defect in intracellular trafficking of the protein. Inhibition of calcineurin activity with cyclosporin A recapitulated the phenotype of CnAα–/– mice, suggesting that phosphatase activity of calcineurin is required for normal AQP2 trafficking and function. As a result, CnAα–/– mice are a new model of nephrogenic diabetes insipidus (NDI) characterized by an impaired response to vasopressin as a result of altered trafficking and phosphorylation of AQP2.

Results

Mice lacking the α isoform of the catalytic subunit of calcineurin (CnAα) were examined to determine the significance of the loss of calcineurin on urine concentration and AQP2 function in vivo. First, urine osmolalities of CnAα wild-type mice (+/+), mice heterozygous for CnAα (+/–) and mice that lack CnAα (–/–) were measured at time points from birth to roughly 4 weeks of age, a time span that includes final post-natal kidney maturation (Fig. 1A). Urine osmolality of +/+ mice increases from 400 mOsm/kg H_2O shortly after birth to a maximum of ~2200 mOsm/kg H_2O at 18 days of age. +/– mice show a slight delay in urine-concentrating ability but reach a normal level of ~2000 mOsm/kg H_2O. By contrast, –/– mice have a defective post-natal urine-concentrating mechanism and obtain a maximum concentration of only ~1400 mOsm/kg H_2O, significantly less than +/+ mice (P<0.001). There was a corresponding significant increase in serum osmolalities in –/– mice (Fig. 1B).

Table 1 summarizes body weight, serum electrolytes, glucose, Ca^{2+}, blood urea nitrogen (BUN), as well a serum and urine creatinine values for each group of mice. –/– mice show a significant decrease in body weight, as previously described by us (Gooch et al., 2004b). There was a trend towards a decrease in serum glucose in +/– mice, and –/– mice were significantly hypoglycemic. In addition, there was a small but significant decrease in Ca^{2+} levels in –/– mice. Renal function – as determined by BUN, serum creatinine and urine creatinine – was slightly abnormal in +/– mice and significantly altered in –/– mice. BUN and serum creatinine were significantly higher and excretion of creatinine in urine was significantly lower in –/– mice compared with +/+ and +/– mice. These results are consistent with impaired renal function as we previously described (Gooch et al., 2004b).

Table 1. Body weight, serum chemistry and urine creatinine values for 18-24 day old CnAα wild-type, heterozygous and knockout animals

	+/+	+/–	–/–
Body weight (G)	10.7±0.3	10.7±0.3	5.3±0.2**
Sodium (Eq/L)	150±1	150±3	147±2
Potassium (Eq/L)	7.3±0.8	6.9±0.4	7.0±0.9
Chloride (Eq/L)	114.4±1.7	113.0±0.8	119.3±3.0
Glucose (Mg/Dl)	181±17	159±11	60±35***
Calcium (Mg/Dl)	11.0±0.5	10.7±0.4	9.4±0.4*
BUN (Mg/Dl)	25±2	29±2	104±34***
Serum creatinine (Mg/Dl)	0.5±0.1	0.8±0.1*	1.7±0.2**
Urine creatinine (Mg/Dl)	23±2	25±3	7±1*

Data shown are the mean ± s.e.m. of 2-8 animals. *P<0.05; **P<0.001, ***P=0.01.

Basal urine concentrating capabilities depend on multiple factors including endogenous vasopressin hormone. However, if the change in basal concentration is due to a defect in circulating hormone, administration of a vasopressin analog should restore concentrating capacity. As such, we injected the vasopressin analog 1-desamino-8D-arginine vasopressin

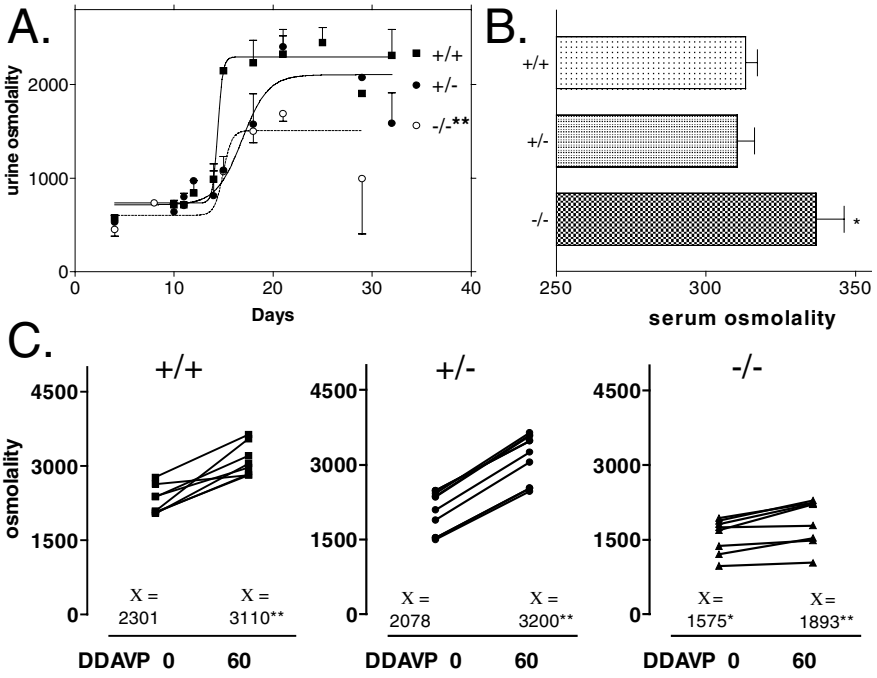

Fig. 1. Urine and serum osmolality. (A) Urine osmolality was measured at multiple time points. Data are shown as the mean ± s.e.m. of 4-10 mice per time point. **P<0.001 compared with +/+ mice (ANOVA). (B) Serum osmolality was measured 18-24 days post-natal. Bars show the mean ± s.e.m. of 4-6 mice. *P<0.05 compared with +/+ mice (Student's t-test). (C) Urine osmolalities of +/+, +/–, and –/– mice were measured before and 60 minutes after DDAVP application. Data shown are ten mice per genotype. *P<0.05 compared with +/+ mice (Student's t-test), **P<0.05 (paired t-test).

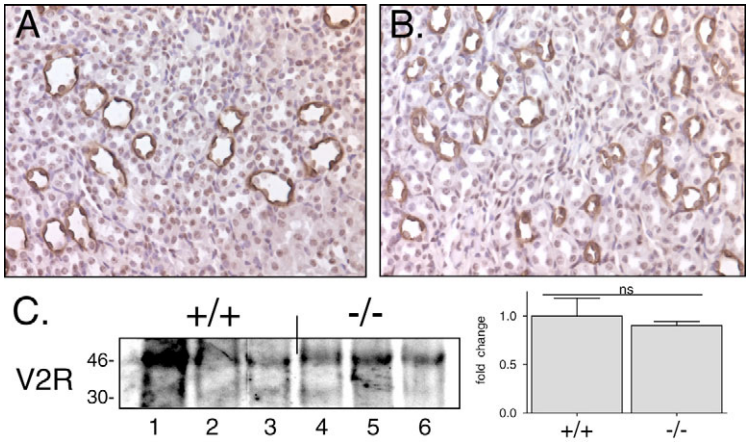

Fig. 2. Expression of the vasopressin receptor in CnAα knockout mice. (A,B) Expression of V_2R was visualized by immunohistochemistry in kidney sections of (A) +/+ and (B) –/– mice using the DAB chromagen and counterstaining with hematoxylin. Data are representative of at least four mice. (C) Expression of the V_2R was also determined by first immunoprecipitating with a specific antibody against the receptor and then immunoblotting with the same antibody. Lanes 1-3, +/+ mice; lanes 4-6, –/– mice. Bar graph on the right shows expression of V_2R as the mean ± s.e.m. ns, not significant.

(DDAVP) to CnAα mice and measured urine osmolality before and 60 minutes after injection (Fig. 1C). As expected, +/+ mice responded to DDAVP with a significant increase in urine osmolality. Despite a slightly lower basal osmolality, +/– mice also responded to DDAVP. By contrast, –/– mice not only have a lower basal urine osmolality compared with +/+ mice ($P<0.01$), but also failed to concentrate to the same extent as +/+ and +/– mice. Although there is a statistically significant increase in urine osmolality after DDAVP injection within the –/– group (paired *t*-test, $P<0.05$), the average increase is significantly less than in +/+ mice ($P<0.05$). Urine output of –/– mice was not obtained because most of the mice survive, on average, only a few days past weaning. However, urine output of +/– mice was determined and found to be significantly increased compared with +/+ littermates [27.8 nl/minute/g body weight compared with 18.3 nl/minute/g body weight ($P<0.05$)].

Owing to the apparent lack of response to DDAVP, we confirmed that the vasopressin type-2 receptor (V_2R), which is activated by DDAVP, is expressed in –/– mice and found that the receptor was similarly expressed in both +/+ and –/– kidneys (Fig. 2). Since exogenous DDAVP failed to restore normal urine-concentrating capacity, we next examined AQP2 directly to determine whether expression and function of the water channel was normal. Maturation of post-natal urine-concentrating ability coincides with increased expression and action of AQP2 (Yamamoto et al., 1997; Yasui et al., 1996). Therefore, we examined expression of AQP2 in CnAα +/+ and –/– kidneys by immunohistochemistry and found that AQP2 is expressed in the cortex and medulla of both genotypes (Fig. 3A,B). Western blot analysis showed that levels of AQP2 are similar in all three genotypes and appear in a pattern characteristic of AQP2: a 34-45 kDa smear of the glycosylated form and a distinct band at approximately 29 kDa of the unglycosylated form (Fig. 3C). AQP2 activation by vasopressin is associated with increased phosphorylation, so levels of phosphorylated AQP2 (p-AQP2) were determined in medullary lysates in response to DDAVP. Fig. 4A shows a significant increase in DDAVP-mediated p-AQP2 in +/+ mice. In contrast, there is no increase in p-AQP2 in CnAα–/– mice following administration of the vasopressin analog (Fig. 4B). AQP2 is phosphorylated by PKA in response to DDAVP (Ausiello et al., 1987; Hayashi et al., 1994). The observed lack of p-AQP2 could be due to alterations in PKA

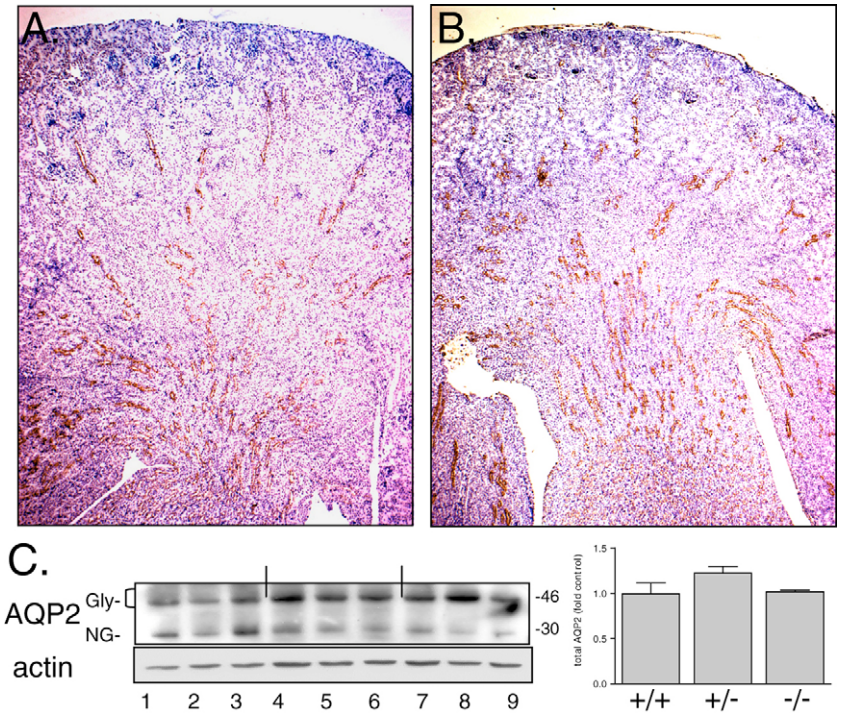

Fig. 3. Characterization of AQP2 in CnAα +/+ and –/– mice. Expression of total AQP2 was visualized by immunohistochemistry in kidney sections of (A) +/+ and (B) –/– mice using the DAB chromagen and counterstaining with hematoxylin. Data are representative of at least four mice. (C) AQP2 expression was determined by western blotting with specific antibodies. Characteristic forms of glycosylated protein (Gly) is observed at 35-45 kDa and non-glycosylated (NG) at 29 kDa. Lanes 1-3, +/+; lanes 4-6, +/–; lanes 7-9, –/–. Expression of actin served as a control. Results from two independent experiments were evaluated by semi-quantitative analysis and results are shown as bar graphs as the mean ± s.e.m.

expression and/or activity in the –/– mice. When examined by western blotting, PKA expression was comparable in +/+, +/– and –/– mice (Fig. 4C). Fig. 4D shows that PKA is activated in +/+ mice in response to DDAVP as expected, but that basal levels of PKA activity are significantly increased in –/– mice and are not further elevated with DDAVP. Consistent with increased basal levels of PKA, we also found that cAMP levels are also constitutively increased with loss of CnAα. Using a commercially available kit (Cayman Chemical, Ann Arbor, MI), we found that there is a 1.48-fold increase in cAMP levels in kidneys of +/– mice (P<0.05) and a 1.84-fold increase in kidneys of –/– mice (P<0.001) compared with wild-type littermates.

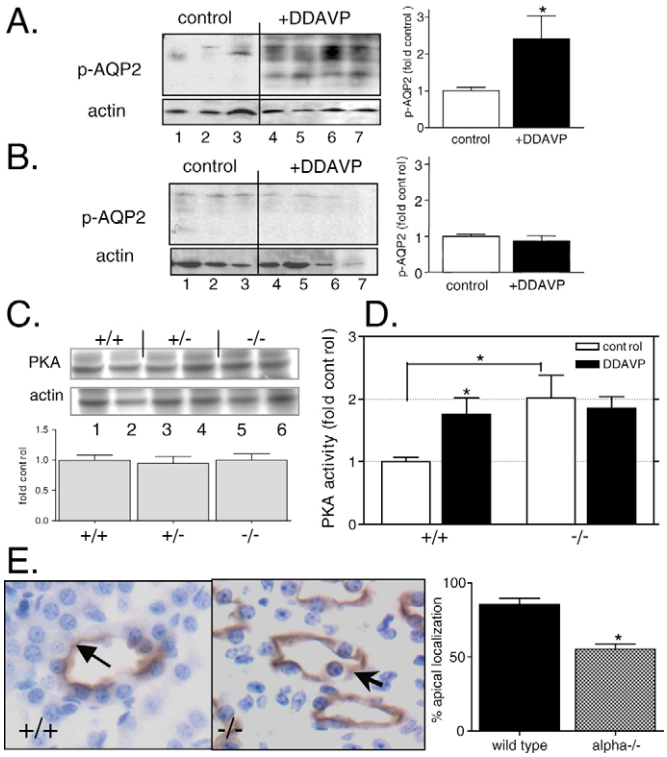

Fig. 4. Phosphorylation of AQP2 by DDAVP in CnAα +/+ and –/– mice. (A) P-AQP2 was detected by immunoblotting with a specific antibody in medullary protein lysates from +/+ mice treated either with vehicle control or DDAVP for 1 hour. Actin was included as a control. Lanes 1-3, untreated; lanes 4-7, plus DDAVP. Results from typical immunoblots were evaluated by semi-quantitative analysis and are shown in bar graph as the mean ± s.e.m. *P<0.05 (Student's t-test). (B) P-AQP2 was detected in medullary protein lysates from –/– mice treated with either control or plus DDAVP for 1 hour. Actin was included as an internal control. Lanes 1-3: control mice; and lanes 4-7: plus DDAVP. Results from typical immunoblots were evaluated by semi-quantitative analysis and results are shown as bar graphs as the mean ± s.e.m. (C) Expression of PKA was determined by western blotting using a specific antibody in +/+, +/– and –/– mouse kidney-cell homogenates. Results from two independent experiments were evaluated by semi-quantitative analysis and are shown as bar graphs as the mean ± s.e.m. (D) In an in vitro assay (see Materials and Methods) activity of PKA was determined in homogenates of whole kidneys of CnAα +/+ and –/– mice that had been either treated with vehicle only (control) or with DDAVP for 1 hour. Bars show the mean ± s.e.m. of 4-6 animals per genotype. *P<0.05 compared with +/+ control (Student's t-test). (E) Intracellular localization of AQP2 was examined by immunohistochemistry in the inner strip of the outer medulla in both +/+ and –/– mice following DDAVP treatment. Arrows identify AQP2 apical localization in +/+ and cellular/baso-lateral distribution in –/– mice. Magnification is 40×. Results of 4-6 mice per group were evaluated by semi-quantitative analysis and are shown in bar graph as the mean ± s.e.m. *P<0.05.

Phosphorylation of AQP2 enhances apical membrane accumulation of the water channel (Christensen et al., 2000; Fushimi et al., 1997; Nishimoto et al., 1999) and impaired phosphorylation of AQP2 may be associated with altered trafficking of the protein (van Balkom et al., 2002). Fig. 4E shows the cellular distribution of AQP2 in DDAVP-treated +/+ and –/– mice as determined by immunohistochemistry. AQP2 is concentrated at the apical surface of IMCD cells in +/+ mice (arrow) but is not found at the apical surface in –/– mice (arrow). Semi-quantitative analysis of apical localization in collecting ducts of wild-type versus knockout mice revealed that less than half of –/– IMCD cells showed primarily apical localization of AQP2 compared with over 80% for +/+ mice. To further identify subcellular localization of AQP2, sections from kidney inner medullae of +/+ and –/– mice were immunogold-labeled for AQP2 and examined by electron microscopy. In +/+ mice, there is extensive labeling of AQP2 in the subapical region as well as some localization to the plasma membrane (Fig. 5A). As expected, after treatment with DDAVP, AQP2 localization in the apical membrane is substantially enriched, (Fig. 5C). By contrast, there is a deficit of AQP2 in the subapical region of –/– mice (Fig. 5B). After injection of DDAVP, there is no accumulation of AQP2 in the apical membrane (Fig. 5D).

AQP2 is processed in the ER and the Golgi network prior to targeting of the molecule to vesicles and the subapical membrane region. Phosphorylation of AQP2 in vesicles is key to enhanced accumulation of AQP2 into the membrane; but trafficking of AQP2 through the ER and Golgi may also be regulated in part by phosphorylation (Procino et al., 2003). By fractionating the cells into vesicle-enriched, cytoplasmic (used as a control, data not shown), and membrane fractions (MFs) (which contain both plasma membrane as well as intracellular membranes such as the ER and Golgi organelles), we determined the subcellular localization of AQP2 in kidneys of wild-type, heterozygous, and knockout mice. To begin, we examined expression of total and p-AQP2 in IMCD vesicle fractions (VF). Loss of both CnAα alleles resulted in a significant decrease in the amount of AQP2 localized in the VF (Fig. 6A). Likewise, p-AQP2 signal in this fraction was decreased with loss of alpha in a dose-dependent manner. Calcineurin has been shown to colocalize with AQP2 in the rat kidney and to be associated with AQP2 in endosomal fractions (Gooch et al., 2004a; Jo et al., 2001). However, activity of calcineurin in association with AQP2-bearing vesicles has not been demonstrated in vivo. Fig. 6B shows that calcineurin protein can be identified in VFs of wild-type mice and that there is a decrease in heterozygous and knockout mice consistent with loss of one and two genes of the α isoform, respectively (the remaining signal is presumably the β isoform because the antibody detects both). Similarly, we find that calcineurin activity that is associated with the VF is decreased in +/– mice and significantly lower (but not completely abrogated) in –/– mice (Fig. 6C). Finally, loss of calcineurin

expression and activity in IMCD VFs corresponded to an increased expression of AQP2 in the MF. Fig. 6D shows that there is only a small amount of total and p-AQP2 in the MF from +/+ mice. In contrast, there is a significant increase in both total and p-AQP2 in +/– mice and an even further increase in the MF of –/– mice.

CnAα–/– mice lack all α-isoform protein and, as a result, suffer from developmental abnormalities and kidney failure (Gooch et al., 2004b). To focus specifically on the role of calcineurin activity in a normal mouse model, we administered cyclosporin A (CsA) to +/+ mice for 3 days and then examined their response to DDAVP. First, activity of CnA was determined in whole-kidney lysates from control mice, mice treated for 3 days with vehicle alone followed by DDAVP for 1 hour (DDAVP), and mice treated with CsA for 3 days followed by DDAVP for 1 hour (CsA+DDAVP) in an in-vitro assay as described (Gooch et al., 2003; Gooch et al., 2001; Gooch et al., 2004b). DDAVP treatment stimulated a significant increase in calcineurin activity (Fig. 7A) compared with controls. By contrast, daily administration of CsA resulted in only a 1.7-fold increase, a change that is significantly less ($P<0.05$) than DDAVP-treated mice and not different from control. Protein levels of total CnA were examined by western blotting and found to be comparable in control, DDAVP and CsA+DDAVP-treated mice (Fig. 7B). Finally, changes in urine osmolality before and 60 minutes after DDAVP were examined (Fig. 7C). Control mice responded to DDAVP with a significant increase in urine osmolality. Whereas basal osmolality was unchanged, inhibition of calcineurin with CsA prevented a significant response to DDAVP.

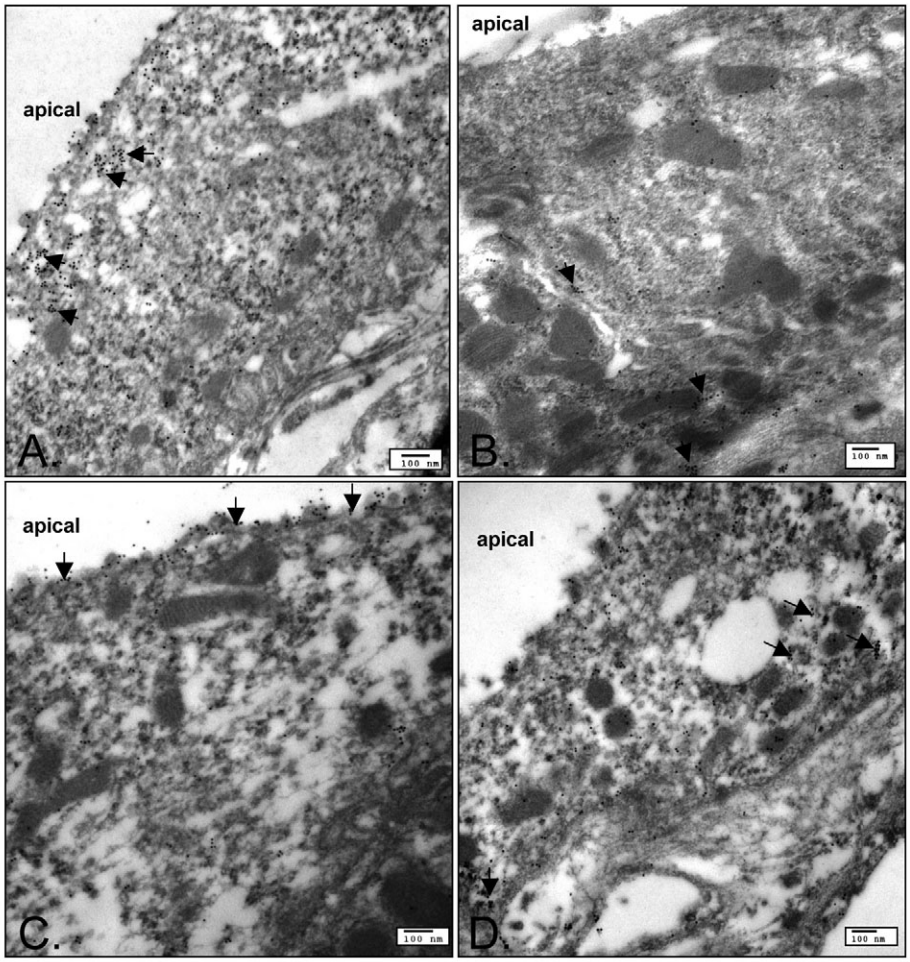

Fig. 5. Subcellular localization of AQP2 in CnAα +/+ and –/– mice. Ultrathin IM sections of kidneys from +/+ and –/– mice were immunogold labeled for AQP2, and viewed by electron microscopy. (A) +/+, (B) –/–, (C) +/+ plus DDAVP, (D) –/– plus DDAVP. Bars, 100 nm.

Next, we examined phosphorylation and localization of AQP2 in control, DDAVP, and CsA+DDAVP-treated mice. First, expression of total-AQP2 and p-APQ2 were determined in the three groups by western blotting (Fig. 8A). Results of immunoblots were analyzed by densitometry and results are shown in bar graphs. Levels of total AQP2 were unchanged with DDAVP or with CsA treatments. As expected, p-AQP2 was significantly higher in DDAVP-treated mice than in the control group ($P<0.05$). However, there was no significant difference between control and CsA+DDAVP treated mice. Moreover, when AQP2 localization was examined by immunohistochemistry there was significantly less migration of AQP2 to the apical membrane of CsA+DDAVP mice ($P<0.05$). Only 35% of medullary collecting ducts of CsA-treated mice displayed predominantly apical localization of AQP2 compared with 82% of collecting ducts of wild-type mice.

Since there appear to be alterations in AQP2 localization with inhibition of calcineurin, we next looked at the effect of CsA on distribution of AQP2 in inner medullary collecting duct cell fractions. Fig. 9A shows expression and phosphorylation of AQP2 in VF. There is a decrease in the amount of total AQP2 and a decrease in p-AQP2 after treatment with CsA. Similar to data with CnAα–/– mice, CsA treatment results in a significant increase in both p-AQP2 and total AQP2 in the MF.

Discussion

In this study, we show that genetic loss of CnAα results in a new model of NDI. CnAα–/– mice fail to develop a mature urine-concentrating mechanism and have an impaired response to DDAVP. This defect is associated with a lack of p-AQP2 in response to vasopressin and a concomitant decrease of AQP2 accumulation in the apical membrane. Further, it appears that loss of calcineurin alters intracellular AQP2 processing such that less AQP2 is correctly routed to vesicles. Rather, there is an increase in AQP2 associated with the cellular MF. Whereas the MF does contain plasma membrane, electron microscopy of immunogold-labeled AQP2 demonstrates that AQP2 is not

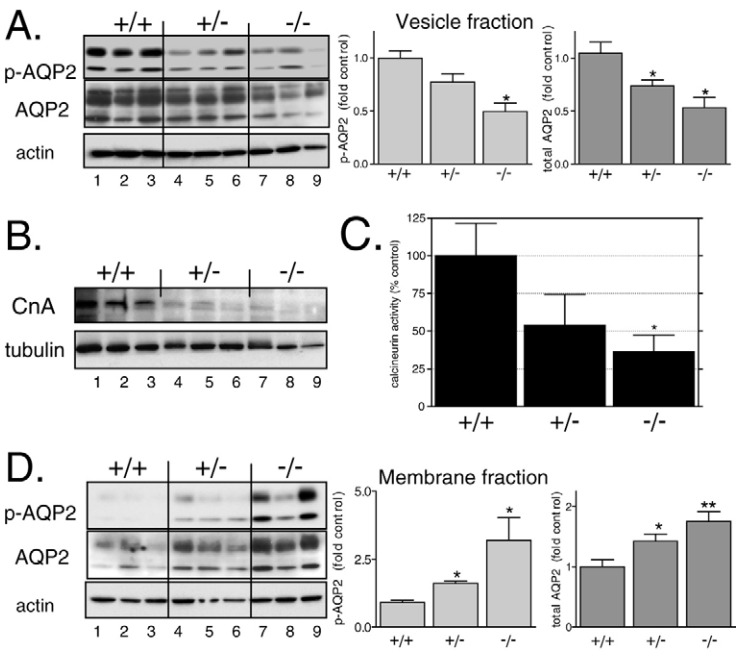

Fig. 6. Expression of total and p-AQP2 in IMCD-cell vesicle and membrane fractions. (A) IMCD-cell vesicle fractions (VFs) were isolated from +/+, +/– and –/– mice. Expression of total and p-AQP2 was detected by immunoblotting with specific antibodies. Actin was included as a control. Lanes 1-3, +/+; 4-6, +/–; and 7-9, –/–. Levels of total and p-AQP2 were evaluated by semi-quantitative analysis, normalized against actin and shown in bar graph. Data show the mean ± s.e.m. of data from 3-5 mice. *P<0.05 (Student's t-test). (B) Calcineurin activity and expression were examined by western blotting VFs with an antibody that crossreacts with the phosphatase domain of all three A subunit isoforms. Actin expression was examined in the same samples as an internal control. (C) Calcineurin activity in isolated VFs was determined in an vitro assay. Bar graphs show the mean ± s.e.m. of 4-6 samples per group. *P<0.01 (Student's t-test). (D) MFs were prepared and expression of total and p-AQP2 were found by direct immunoblotting. Lanes 1-3, +/+; lanes 4-6, +/–; lanes 7-9, –/–. Actin was included as a control. Levels of total and p-AQP2 were evaluated by semi-quantitative analysis and results are shown in bar graphs as the mean ± s.e.m. of data from 3-5 mice. *P<0.05, **P<0.01 (Student's t-test).

in the apical membrane with loss of calcineurin, thus it is likely that AQP2 is retained in intracellular membrane compartments, such as the ER or the Golgi network. As a result, there is a significant decrease in the amount of AQP2 available for immediate activation by DDAVP. Fig. 6A shows that phosphorylation of AQP2 in vesicles of CnAα null mice is roughly comparable to expression of total AQP2, suggesting that AQP2 – which is localized to the vesicles – can be normally phosphorylated. Consequently, the defect in AQP2 signaling that results in NDI in CnAα–/– mice is the result of a defect prior to vesicularization of AQP2. Accumulation of AQP2 in the membrane fraction but not the apical cell membrane support the conclusion that loss of calcineurin impairs routing of AQP2 prior to vesicle trafficking.

Several considerations must be made when interpreting physiological studies in these mice. First, the majority of –/– mice die between 24 and 28 days of age. Thus, studies were focused on days 18-24 post-natal because the concentrating mechanism is mature in +/+ mice, but the average lifespan limit of –/– mice has not been reached. As we previously reported, CnAα–/– mice have noticeable defects in kidney development, particularly in late-developing nephrons within the outer stripe of the outer medulla and the cortex (Gooch et al., 2004b). The lack of cortical mass might, in fact, contribute to a lower basal urine osmolarity in CnAα–/– mice because proximal tubule reabsorption is required for normal urine concentrating (Nielsen et al., 2002). These mice also suffer from renal failure, as evidenced by elevations in BUN and serum creatinine (Table 1). In light of these limitations, this study was expanded to include +/+ mice treated with CsA for 3 days to inhibit calcineurin function. Interestingly, pre-treatment of mice with CsA resulted in two differences compared with CnAα mice. First, basal concentrating capacity of CsA-treated mice was unchanged after 3 days of treatment, whereas there is a significant decrease in basal urine osmolality in CnAα–/– mice compared with wild-type littermates. However, in studies of

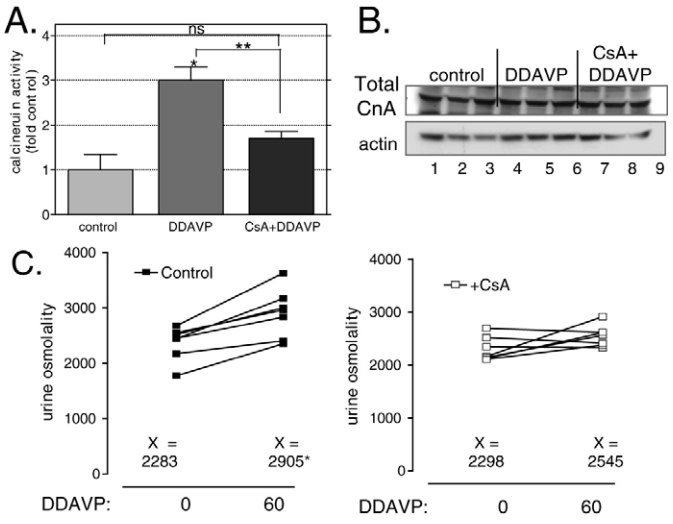

Fig. 7. Effect of CsA on calcineurin expression and activity and on response to DDAVP. (A) In an vitro assay (see Materials and Methods), activity of calcineurin was determined in homogenized whole kidneys from control, DDAVP, and CsA+DDAVP mice. *P<0.05 compared with wild-type, **P<0.05 compared with DDAVP-treated mice. Bar graphs show the mean ± s.e.m. (B) Expression of total CnA was determined in protein from homogenized whole kidneys of control, DDAVP and CsA+DDAVP animals by direct immunoblotting with specific antibodies. Actin was included as a control. Each lane contains protein isolated from one animal. Lanes 1-3, control; lanes 4-6, DDAVP; lanes 7-9: CsA + DDAVP. (C) Urine osmolality was measured in control and CsA-treated animals before and 60 minutes after the subcutaneous injection of DDAVP. Data shown are from eight individual animals per treatment group. *P<0.05 (paired t-test). Also shown is the mean osmolality of each group.

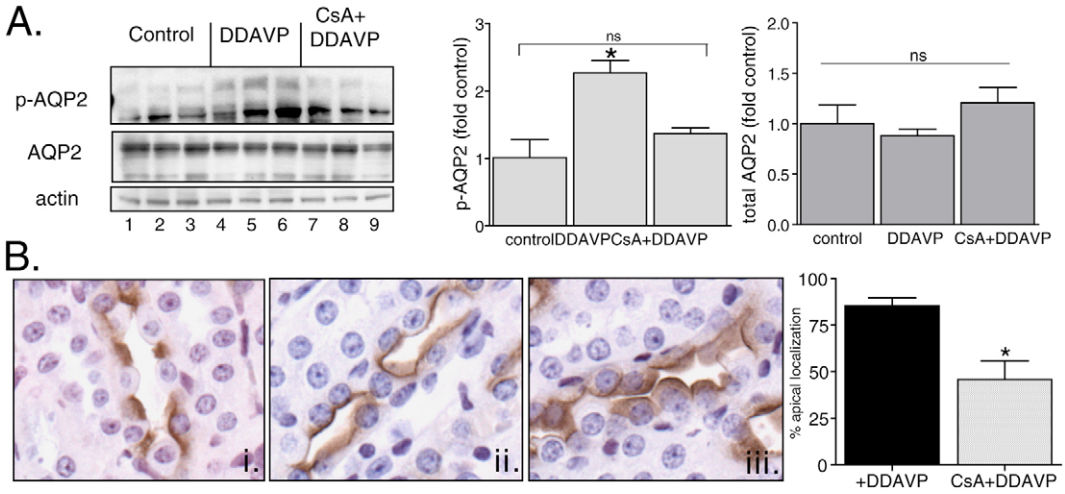

Fig. 8. Effect of CsA on DDAVP-mediated regulation of AQP2. (A) Expression of p-AQP2 and total AQP2 was determined in whole kidney lysates of control, DDAVP, and DDAVP + CsA-treated mice by immunoblotting with specific antibodies. Actin was included as a control. Lanes 1-3, control; lanes 4-6, DDAVP; lanes 7-9, CsA + DDAVP. Amounts of total and p-AQP2 were evaluated by semi-quantitative analysis and results are shown in bar graphs. (B) AQP2 localization was detected by immunohistochemistry in kidney IM sections with the DAB chromagen and sections were counterstained with hematoxylin. (i) Control, (ii) DDAVP, (iii) DDAVP + CsA. Shown are typical data of three mice per group. Magnification is 40× Results of immunohistochemistry were evaluated by semi-quantitative analysis and are shown in as the mean ± s.e.m. *P<0.05.

long-term CsA administration, basal urine concentration did decrease (Lim et al., 2004), in conjunction with downregulation of AQP2 as well as other aquaporins and urea transporters. Second, wild-type mice treated with CsA demonstrated even less urine-concentrating response to DDAVP than CnAα–/– mice. This result suggests that the CnAβ isoform remains active in CnAα–/– mice and contributes to vasopressin-mediated urine-concentration, albeit at a lower rate than the α isoform. Likewise, we found that calcineurin activity is substantially reduced (>70%) in isolated CnAα–/– IMCD-cell vesicles, but not completely abrogated (Fig. 6C). Compensatory action of CnAβ might also explain why CnAα–/– mice, although similar to models of AQP2 mutation, have a less severe phenotype. Both CnAα–/– and AQP2 mutant mice are born relatively normal but exhibit failure to thrive shortly after birth. CnAα–/– mice survive an average of 26 days whereas AQP2 mutant mice survive about 6 days (Yang et al., 2001). Finally, it is interesting to notice that regulation of AQP2 by CnAα appears to be dose-dependent. +/– mice have an intermediate phenotype, including mild polyuria, and a slightly lower basal urine-concentrating capacity. In addition, +/– mice also display an intermediate phenotype with regard to retention of AQP2 in the MF and a resulting decrease in AQP2 in IMCD-cell vesicles. Interestingly, this is accompanied by a relative increase in p-AQP2 in the VF, explained, perhaps, by slightly higher PKA activity (our unpublished data) and higher basal levels of cAMP. Taken together, these data suggest a compensatory effort to increase vasopressin signaling and AQP2 activation in the face of impaired routing of AQP2 to the membrane. Likewise, in CnAα–/– mice, the significant increases in basal cAMP and PKA activity might be the result of a compensatory effort to restore normal vasopressin responsiveness ,and also suggest that these components of the pathway can be regulated in the expected manner, again pointing to AQP2 trafficking as the primary defect.

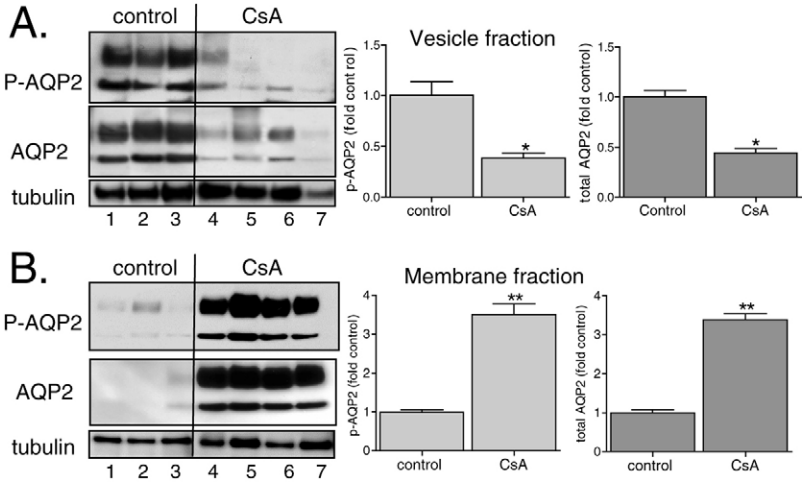

Fig. 9. Effect of CsA on distribution of VF and MF, and phosphorylation of AQP2. (A) IMCD-cell VFs were isolated from control and CsA-treated mice. Expression of total and p-AQP2 was detected by immunoblotting with specific antibodies. Tubulin was included as a control. Lanes 1-3, control; lanes 4-7, CsA-treated mice. Levels of total and p-AQP2 were evaluated by semi-quantitative analysis and data from 3-5 mice are shown as the mean ± s.e.m. *P<0.05 (Student's t-test). (B) MFs were prepared and expression of total and p-AQP2 was detected by direct immunoblotting. Tubulin was included as a control. Lanes 1-4, control; lanes 5-8, CsA-treated. Levels of total and p-AQP2 were evaluated by semi-quantitative analysis and data from 3-5 mice are shown as the mean ± s.e.m. *P<0.05, **P<0.01 (Student's t-test).

One significant finding of the study is the implication of calcineurin in regulation of intracellular processing of AQP2. Prior to the sub-apical localization of AQP2-bearing vesicles, the water channel must be processed through the ER and Golgi network. The importance of this processing step is highlighted by the fact that a number of inherited mutations in the AQP2 gene result in proteins that are retained in the ER (Knoers and Deen, 2001). Interestingly, some of these mutations might involve Ser256, the residue that is phosphorylated by PKA prior to enhanced accumulation of AQP2 into the apical membrane. Whereas loss of Ser256 itself results in constitutive vesicularization of AQP2 and does not result in intracellular accumulation (van Balkom et al., 2002), loss of specific residues in close proximity to Ser256, including Glu258Lys does (Hirano et al., 2003). This suggests that there might be differential regulation of the phosphorylation at Ser256, such that two different functions of the protein can be altered – membrane accumulation subsequent to PKA activity or intracellular trafficking – depending on which interaction is disrupted. This was demonstrated by Procino et al., who reported that AQP2 was weakly phosphorylated at Ser256 during normal AQP2 trafficking in ER and that phosphorylation increased during transit to the Golgi in a PKA-independent manner (Procino et al., 2003). Interestingly, Procino et al. also found that exit of AQP2 from the Golgi and into vesicles was associated with dephosphorylation of AQP2. When considering the finding of Jo et al. that calcineurin co-immunoprecipitates with AQP2-bearing vesicles and dephosphorylates AQP2 in vitro (Jo et al., 2001), and our previous result that CnAα colocalizes with AQP2 in IMCD cells in vivo, it is enticing to suggest that CnAα regulates AQP2 trafficking by dephosphorylating the protein during Golgi and/or vesicular routing. Our finding that loss of calcineurin protein and activity associated with VFs is associated with increased accumulation of AQP2 in the MF supports this conclusion. We also show that CnAα is activated acutely by vasopressin, thus it is interesting to speculate that CnAα also contributes to membrane shuttling of AQP2. However, our data thus far do not demonstrate in which intracellular membrane compartment AQP2 is retained. Future experiments will be necessary to identify the exact step in intracellular processing that is disrupted by loss of CnAα.

Our finding that CnAα participates in the regulation of AQP2 is significant not only because it defines a new element required for normal processing of AQP2, but also because our data suggest a role for CnAα in protein processing. Calcineurin is a Ca^{2+}-regulated protein and has been shown to be associated with the Ca^{2+} channels of the ER, namely the inositol (1,4,5)-trisphosphate receptor [Ins(1,4,5)P_3] and the ryanodine receptor (RyR) (Cameron et al., 1995). Moreover, recent evidence that calcineurin might be involved in intracellular processing comes from calreticulin-deficient mice. Rauch et al. originally reported that mutation of the ER chaperone was embryonic lethal (Rauch et al., 2000). But when calreticulin+/– mice were crossed with mice carrying a constitutively active calcineurin gene that is expressed specifically in the heart, calreticulin–/– mice were partially rescued (Guo et al., 2002). From this experiment, it can be concluded that calcineurin function – in particular in the developing heart – is required for normal protein processing through the ER chaperone system. Our data supports this idea as well. AQP2 expression is developmentally regulated (Nielsen et al., 2002) and increases at 2 to 3 weeks of age, coordinating with final post-natal maturation of the kidney. Loss of CnAα does not alter AQP2 expression, but severely impairs intracellular processing of the protein. Moreover, we show that retention of AQP2 requires calcineurin dephosphorylation of a substrate because CsA treatment of +/+ IMCD cells produces the same effect.

NDI is a disorder characterized by renal unresponsiveness to vasopressin; the vasopressin-regulated pathway is disturbed most commonly by loss of functional V_2R or AQP2. Our data suggest that an additional mechanism for development of NDI is via the disruption of calcineurin expression or activity. In particular, the possibility that pharmacological inhibition of calcineurin results in defects in urine concentration is an important area of future investigation.

Materials and Methods

Materials

Total CnA antibody was obtained from Chemicon (Temecula, CA), anti-actin, anti-PKA, and anti-CnAα antibodies were from Santa Cruz. Anti-AQP2 antibody was a gift of Janet Klein (Emory University, Atlanta, GA) and an antibody generated against Ser256 of AQP2 was a gift of Soren Nielsen (University of Aarhus, Aarhus, Denmark) (Christensen et al., 2000; van Balkom et al., 2002). Cyclosporin A (CsA) (Sangstat) was obtained from Eli Lilly Corporation (Indianapolis, IN) and DDAVP from Sigma-Aldrich (St Louis, MO).

Transgenic mice

Mice were created by J. Seidman (Harvard Medical School, Boston, MA) as previously described (Zhang et al., 1996) and provided to our laboratory. Mice were maintained and bred at the Audie Murphy Veterans Hospital, San Antonio, in accordance with IACUC standards. Most CnAα–/– mice die between 3 and 4 weeks of age (personal communication J. Seidman) (see also Gooch et al., 2004b). Therefore, unless otherwise stated, studies described here were conducted between 18 and 24 days of age. CnAα–/– mice were created on a mixed genetic background (Zhang et al., 1996). Therefore, all experiments were carried out with +/+ and, where indicated, +/– littermates. For some experiments, +/+ mice were divided into three groups and treated as follows: group 1 (control); group 2 (DDAVP) received a daily s.c. injection of 10% ethanol in saline (vehicle control) for 3 days and then a single i.p. injection of 1 μg/kg desmopressin acetate (1-desamino-8D-arginine-vasopressin, DDAVP); group 3 (CsA+DDAVP) received a daily s.c. injection of 10 mg/kg CsA for 3 days and then a single i.p. injection of 1 μg/kg DDAVP.

Urine was collected manually with abdominal massage or by housing the mice individually in metabolic cages. Blood was collected by cardiac puncture at the time of sacrifice. Serum chemistries were performed by the University Health System Pathology Services Referral Laboratory (San Antonio, TX) using an Olympus 640e chemistry analyzer; osmolalities were measured using a vapor-pressure osmometer (Wescor Instruments).

IMCD fractionation

IMCD vesicles and MFs were derived by differential centrifugation as described (Fernandez-Llama et al., 1998; Tamma et al., 2003). Briefly, kidneys were harvested and immediately placed in isolation solution (IS) (10 mM triethanolamine plus 250 mM sucrose) plus protease inhibitors [100 μg/ml leupeptin, 20 μg/ml aprotonin, and 10^{-7} M phenylmethylsulfonyl (PMSF)]. Medullae and cortices were dissected, minced into small pieces with a razor, and then homogenized by douncing 50 times in IS. For some experiments, dissected cortices and medullae were retained for extraction of total protein. In other experiments, homogenized medullae were centrifuged at 1000 ***g*** for 10 minutes and the supernatants transferred to new tubes. The supernatants were then centrifuged at 4000 ***g*** for 20 minutes at 4°C, to pellet mitochondria and nuclei. Pellets were discarded and supernatants were then centrifuged at 17,000 ***g*** for 20 minutes at 4°C to isolate a pellet consisting primarily of plasma membrane, Golgi network and ER. These pellets (17,000 ***g*** pellets) were resuspended in lysis buffer and retained as the MF. The resulting supernatant was centrifuged at 100,000 ***g*** for 1 hour at 4°C. The 200 k pellet, which is enriched for vesicles, was resuspended in isolation solution and designated the VF. These supernatants (100,000 ***g*** supernatants) were retained as a negative control. Content of the fractions was verified by western blotting, with antibodies against translation factors as a control for the ER and against AQP2 as a control for vesicles.

Calcineurin phosphatase assay

Lysates were prepared by resuspending dissected kidneys in a hypotonic lysis buffer (50 mM Tris pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5 mM DTT, 50 μg/ml PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin) followed by dounce homogenization and three cycles of freeze-thawing in liquid nitrogen and a 30°C water bath. Calcineurin

phosphatase activity was determined as previously described (Fruman et al., 1996; Gooch et al., 2003; Gooch et al., 2001). Control reactions were simultaneously performed in a reaction buffer in which EGTA was substituted for $CaCl_2$. Final calcineurin activity was calculated by subtracting the Ca^{2+}-independent activity in EGTA buffer from each reaction and normalizing against μg protein.

PKA assay

PKA activity in cell or kidney lysates was determined according to the manufacturer's protocol (CalBiochem, San Diego, CA). Briefly, cells or tissue were homogenized in a lysis buffer containing 25 mM Tris-HCl pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton X-100, 10 mM β-mercaptoethanol, 1 μg/ml leupeptin and 1 μg/ml aprotinin. Lysates were then incubated in a reaction mixture that includes a biotinylated peptide substrate and [^{32}P]-ATP. The reaction was initiated by addition of 5 μl of PKA sample and terminated by addition of 50% trichloroacetic acid solution and 1% bovine serum albumin. Avidin solution was added to bind to the biotinylated-^{32}P-peptide product. The samples were then centrifuged through a column that retained the product-avidin complex. The retained sample was counted in a scintillation counter to determine the extent of PKA activity in the sample. All experiments were performed with controls, including reaction without enzyme, reaction mix without sample and a control reaction without the sample peptide.

Western blotting

Whole kidneys, dissected inner medullae or cortices, inner medullary collecting duct VFs or cellular membrane fractions were homogenized in TNESV (50 mM Tris-HCl pH 7.4, 2 mM EDTA, 1% NP-40, 100 mM NaCl, 100 mM Na orthovanadate, 100 μg/ml leupeptin, 20 μg/ml aprotonin, and 10^{-7} M PMSF) and western blotting was carried out as previously described (Gooch et al., 2004b).

Histology

Kidneys were immersed in 10% neutral-buffered formalin or quick-frozen in liquid nitrogen for further analyses. Immunohistochemistry and immunofluorescence were carried out as previously described (Gooch et al., 2004a). For immuno-gold labeling, medullae were dissected, finely chopped, fixed in 4% paraformaldehyde with 0.2% picric acid, and then embedded in LR white resin. Sections were cut ultrathin and mounted on nickel grids. Grids were blocked with 1 mg/ml goat IgG in PBS plus 0.05% saponin for 1 hour at room temperature, incubated with rabbit anti-AQP2 (1:1000) overnight, washed three times in PBS-saponin, incubated with 15-nm gold-conjugated goat anti-rabbit IgG for 1 hour, washed a final three times in PBS-BSA, counterstained with 0.5% osmium, 1-2% uranyl acetate and rinsed in de-ionized water. Bound gold particles were visualized with a Philips electron microscope.

Statistics

Statistical analyses were performed by using two-tailed Student's *t*-test unless otherwise noted. A result of $P<0.05$ was considered significant. For some experiments, two-way ANOVA and paired *t*-tests were performed as indicated using GraphPad Prism™ software and results were considered significant if $P<0.05$.

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