Expression of a dominant negative PKA mutation in the kidney elicits a diabetes insipidus phenotype

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Gilbert ML, Yang L, Su T, McKnight GS. Expression of a dominant negative PKA mutation in the kidney elicits a diabetes insipidus phenotype. Am J Physiol Renal Physiol 308: F627–F638, 2015. First published January 13, 2015; doi:10.1152/ajprenal.00222.2014.—PKA plays a critical role in water excretion through regulation of the production and action of the antidiuretic hormone arginine vasopressin (AVP). The AVP prohormone is produced in the hypothalamus, where its transcription is regulated by cAMP. Once released into the circulation, AVP stimulates antidiuresis through activation of aquaporin-2 receptors in renal principal cells. Vasopressin 2 receptor activation increases cAMP and activates PKA, which, in turn, phosphorylates aquaporin (AQP)2, triggering apical membrane accumulation, increased collecting duct permeability, and water reabsorption. We used single-minded homolog 1 (Sim1)-Cre recombinase-mediated expression of a dominant negative PKA regulatory subunit (RΙβ) to disrupt kinase activity in vivo and assess the role of PKA in fluid homeostasis. RΙβ expression gave rise to marked polydipsia and polyuria; however, neither hypothalamic Avp mRNA expression nor urinary AVP levels were attenuated, indicating a primary physiological effect on the kidney. RΙβ mice displayed a marked deficit in urinary concentrating ability and greatly reduced levels of AQP2 and phospho-AQP2. Dehydration induced Aqp2 mRNA in the kidney of both control and RΙβ-expressing mice, but AQP2 protein levels were still reduced in RΙβ-expressing mutants, and mice were unable to fully concentrate their urine and conserve water. We conclude that partial PKA inhibition in the kidney leads to posttranslational effects that reduce AQP2 protein levels and interfere with apical membrane localization. These findings demonstrate a distinct physiological role for PKA signaling in both short- and long-term regulation of AQP2 and characterize a novel mouse model of diabetes insipidus.

IN RESPONSE to hypernatremia or hypovolemia, arginine vasopressin (AVP) is released into the circulation from the posterior pituitary and acts on renal vasopressin 2 receptors (V2Rs) to promote water reabsorption. Long-term regulation occurs over a period of minutes through V2R activation, generation of cAMP, and insertion of aquaporin (AQP2)-containing endosomes into the apical plasma membrane (44). PKA phosphorylates AQP2 on Ser256, an event that may be required for subsequent phosphorylation at Ser269 by other kinases, and these events lead to insertion and retention of AQP2 into the apical membrane (23). Long-term regulation, as seen with dehydration, occurs over the course of hours to days through increased Aqp2 mRNA levels, leading to a large increase in AQP2 protein (15, 21). Phosphorylation of the cAMP response element (CRE)-binding protein (CREB) is thought to regulate Aqp2 mRNA levels through CREs, which have been identified in the promoter region of the Aqp2 gene (26, 37, 64). The role of PKA in long-term regulation of AQP2 abundance, however, remains unclear as AQP2 expression and collecting duct water permeability remain high in the dehydrated state despite low levels of cAMP, pointing to potential PKA-independent regulation of AQP2 protein levels (32). These factors highlight critical gaps in our knowledge of the regulation of AQP2 protein in the renal principal cell.

The PKA holoenzyme is a tetramer composed of a regulatory (R) subunit dimer that sequesters two catalytic (C) subunits in the absence of cAMP (39). Upon cAMP binding, the C subunits dissociate from the holoenzyme and phosphorylate a range of diverse substrates to regulate cellular functions. To develop mouse genetic approaches that could be used to inhibit PKA in specific cell types, we cloned mutant forms of the RΙβ subunit that suppress C subunit activity even in the presence of physiological levels of cAMP (11, 56). One of these dominant negative mutants (RΙβ) contains a single amino acid change (G324D) in the COOH-terminal cAMP-binding site (site B), a mutation that increases the Kd for activation of the PKA holoenzyme ~100-fold (62). We generated mice in which the RΙβ mutation was introduced into one allele of the Prkarl gene, and a loxP-flanked neomycin resistance cassette was placed between the last two exons to act as a stop signal and prevent expression of the RΙβ allele. Cre-mediated recombination removes this stop cassette and activates the dominant negative RΙβ allele in a cell type-specific pattern (25, 60).

To analyze the role of PKA activity in fluid homeostasis in vivo, we used a transgenic mouse line expressing Cre recombinase to activate cell type-specific expression of RIβ. The single-minded homolog 1 (Sim1)-Cre transgene (2) initiated RΙβ expression in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus as well as AQP2-expressing principal cells of the kidney. Expression of RIβ in the hypothalamic PVN and SON did not appear to affect the transcription of Avp mRNA or release of AVP into the circulation. However, Sim1-directed inhibition of PKA activity in the kidney leads to severe polydipsia, decreased AQP2 protein levels, and a blunted response to short- and long-term V2R activation. A dramatic decrease in AQP2 expression occurred in the RIβ-expressing kidney without alterations in Aqp2 mRNA levels, indicating a prominent role of PKA in regulating posttranslational stability of the water channel in vivo.

EXPERIMENTAL PROCEDURES

Experimental animals. The generation and genotyping of RΙβ mice have been previously described in detail (25, 60). Dr. Brad Lowell (Beth Israel Deaconess Medical Center)
kindly provided Sim1-Cre mice (2). Mice used in this study were housed on a 12:12-h light-dark cycle and had free access to standard chow and water except as noted in the dehydration experiments. All experiments were conducted using protocols approved by the Institutional Animal Care and Use Committee of the University of Washington.

Urine collection and osmolality measurements. Urine production and osmolality were determined in mice with free access to water or after 24-h dehydration. Additionally, mice were subjected to acute water loading (10 mM glucose solution, 3% of body weight, oral gavage) and subsequent intraperitoneal injection of vehicle or the V2R agonist desmopressin (DDAVP; Ferring Pharmaceuticals). For urine collection, mice were placed in a modified metabolic chamber on a wire platform, and spontaneously voided urine was collected over the next 2–4 h. Urine osmolality was determined using a Vapro 5520 vapor pressure osmometer (Wescor).

Urine AVP and cAMP measurements. Spontaneously voided urine was collected as described above, and urine AVP levels were assessed by radioimmunoassay as per the manufacturer’s instructions (ALPCO). For cAMP measurements, mice were given an intraperitoneal injection of DDAVP as described above, and urine samples were analyzed for cAMP content by ELISA (Enzo Life Sciences) as per the manufacturer’s instructions.

X-gal staining and immunohistochemistry. After CO2 euthanasia, mice were perfused transcardially with PBS followed by PBS-buffered 4% paraformaldehyde solution. Tissues were removed, postfixed for 2–4 h in PBS-paraformaldehyde, and then cryopreserved overnight in a 30% sucrose solution (wt/vol). After being embedded in Tissue-Tek OCT compound (Sakura Finetek), 20-μm cryosections were taken and processed for X-gal staining or immunohistochemistry. For X-gal staining, sections were washed with PBS and then incubated with X-gal solution (40 mg/ml X-gal stock solution in DMSO) diluted 1:40 into X-gal buffer [5 mM potassium ferricyanide (crystalline), 5 mM potassium ferricyanide trihydrate, and 2 mM magnesium chloride] at 37°C for 1 h to overnight in PBS. Sections were washed in PBS, dehydrated in 100% ethanol, and counterstained with eosin Y. Sections were mounted on slides with Cytoseal 60 (Thermo Scientific) and imaged by light microscopy on a Nikon Eclipse E600 microscope. For immunohistochemistry, sections were washed in PBS and then incubated in blocking buffer (10% normal goat serum, 0.2% Triton X-100, and 2% BSA in PBS) for 1 h at room temperature. Primary antibodies (1:250) were diluted into blocking buffer, and sections were incubated at 4°C overnight. After being rinsed with PBS with 0.2% Triton X-100, sections were incubated for 2 h at room temperature with fluorescence-labeled secondary antibodies (Molecular Probes, 1:500). Sections were washed in PBS with 0.2% Triton X-100 and mounted with Vectashield medium for imaging on a Leica SL confocal microscope. All images were taken at the Keck Microscopy Facility (University of Washington).

Protein sample preparation and Western blot analysis. After a brief sonication (Branson Sonifier), samples were cleared at 10,000 g for 10 min at 4°C, and the supernatant protein concentration was determined by a BCA assay (Pierce). Protein samples were diluted in 4X NuPAGE sample buffer (Invitrogen), resolved on 12% polyacrylamide gels, and subsequently transferred to nitrocellulose membranes (Whatman). Membranes were blocked for 1 h in 5% BSA or milk in Tris-buffered saline with 0.1% Tween 20 (TBST) and incubated overnight in primary antibody. Membranes were then rinsed with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) diluted at 1:10,000 in TBST with 5% BSA or milk. Horseradish peroxidase was detected with Supernignal West Pico chemiluminescent substrate (Pierce).

Kinase activity measurements. PKA activity was determined by phosphorylation of a PKA substrate peptide (Kemptide, LRRASLG) using [γ-32P]ATP. Tissue samples were collected and dounce homogenized in kinase activity buffer (20 mM Tris, 0.1 mM EDTA, 0.5 mM EGTA, 10 mM DTT, 5 mM magnesium acetate, and 250 mM sucrose; pH 7.6 with 1% Triton-X 100) supplemented with 1 μg/ml leupeptin, 3 μg/ml aprotinin, and 5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF). Total enzymatic activity is expressed as pico-moles of phosphate transferred (in units) per milligram of input protein. Activity measured in the presence of 4 μg/ml protein kinase inhibitor (PKI) was assumed to be nonspecific and subtracted from each sample as background.

Immunoprecipitation of RiboTag-labeled polypeptides from mouse tissues. Mice were euthanized by CO2, and hypothalami or whole kidneys were collected and immediately processed as previously described (51, 52).

Real-time quantitative RT-PCR. Total RNA was isolated from the hypothalamus or kidney using an RNasy RNA isolation kit (Qiagen). One-step RT-PCR was performed using the Mx3000P QPCR system and Brilliant II QRT-PCR reagent kit (Agilent). Relative amounts of transcripts from each tissue were extrapolated from a four-point standard curve (100, 10, 1, and 0.1 ng total RNA) made from a common brain or kidney sample. To ensure equivalent loading of total RNA, β-actin (Actb) was assessed in parallel during each RT-PCR experiment. Primer sequences for each transcript were obtained using PrimerBank (55) and are shown in Table 1.

Primary antibodies. All antibodies were used at 1:1,000 for Western blot analysis and 1:250–500 for immunohistochemistry. Antibodies purchased were as follows:: PKA Co and Rlα subunits (mouse, BD Biosciences), PKA Rlα (rabbit, Santa Cruz Biotechnology), β-actin (mouse, Sigma), AVP (rabbit, EMD Biosciences), hemagglutinin (HA; mouse, Covance), and AQP2 (rabbit, Novus Biologicals). Phospho-AQP2 (Ser256) antibody was a kind gift from Dr. Mark Knepper (National Heart, Lung, and Blood Institute).

Data analysis. Statistical analyses were carried out using Prism 4.0e for the Mac OS (GraphPad Software). Data are presented as means ± SE, and statistical significance was determined by one-way ANOVA between wild-type (WT), Prkar1α<sup>WT</sup>/Rlα<sup>B</sup> (Rlα<sup>B</sup>-OFF), and Sim1-Cre/Prkar1α<sup>WT</sup>/Rlα<sup>B</sup> (Rlα<sup>B</sup>-ON) mice. Where appropriate, a Bonferroni’s multiple-comparison test was conducted, and differences were considered significant at \( P < 0.05 \).
Table 1. Primer sets used for RT-PCR analysis

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RESULTS

RloxB expression directed by Sim1-Cre causes diabetes insipidus in mice. As shown in Fig. 1A, RloxB-OFF mice express only one WT Prkar1a allele, and a loxP-flanked neomycin cassette placed between exons 10 and 11 disrupts RloxB mutant allele (G324D) expression. The Sim1-Cre recombinase transgenic mouse line was crossed to the RloxB line to initiate expression in Sim1-positive cell types. Sim1 is highly expressed in specific nuclei of the hypothalamus, primarily the PVN and SON (2). Previously, it has also been demonstrated that Sim1 is expressed in peripheral tissues during early embryonic development, including cell populations that give rise to the pronephros and primitive metanephros (16, 40). Sim1-Cre-mediated recombination removed the NEO stop cassette, allowing for the resumption of splicing between exons 10 and 11 and initiation expression of mutant RloxB protein. The RloxB-OFF mice (Prkar1aWT/RloxB, Fig. 1B) used in this study are effectively heterozygous for Prkar1a and are included as controls for comparison with WT and RloxB-ON mice (Sim1-Cre/Prkar1aWT/RloxB, Fig. 1B) expressing the mutant protein.

The PVN is a critical regulatory site for water balance as well as energy homeostasis, receiving input from orexigenic (neuropeptide Y/agouti-related peptide) and anorexigenic (pro-opiomelanocortin) neurons in the arcuate nucleus (12). Pro-opiomelanocortin neurons are thought to suppress feeding behavior through activation of melanocortin-4 receptors and increased cAMP generation. Phenotypic analysis of RloxB-ON mice and littermate controls revealed no significant difference in food intake (Fig. 1C) or reproductive fat pad weight (Fig. 1D), but RloxB-ON mice developed a significant increase in body weight by 8 wk of age (change in body weight in male mice: 15.5% and change in body weight in female mice: 22.8%; Fig. 1E). In contrast to the small effects of RloxB activation on these parameters, we observed a marked increase in water consumption in male and female mice (Fig. 1F), a phenotype that was readily apparent by 6 wk of age. In conjunction with increased fluid intake, RloxB-ON mice displayed pronounced polyuria compared with WT and RloxB-OFF littermates (Fig. 1G).

Sim1-Cre directs RloxB expression to selective regions of the hypothalamus and kidney. To follow Sim1-directed Cre recombinase activity in the hypothalamus and kidney, we crossed Sim1-Cre mice to the ROSA26 (R26R) reporter mouse line (54). Cryosections from the brain and kidney were stained with X-gal to reveal β-galactosidase expression. As expected, Sim1-Cre induced reporter activation in the PVN and SON of the hypothalamus, as shown in Fig. 2A, left. β-Galactosidase expression was limited in the renal cortex, whereas higher expression levels were consistently observed in the outer medulla as well as subpopulations of cells interspersed throughout the inner medulla and renal papilla (Fig. 2A, right).

We next used the Ribotag mouse (51, 52) to identify specific cell populations in the hypothalamus and kidney that were targeted by Sim1-Cre. The Ribotag mouse has a modified exon 4 inserted into the ribosomal protein L22 (Rpl22) gene that incorporates three sequential HA epitopes into RPL22 that incorporates three sequential HA epitopes into RPL22 gene and was targeted by Cre-mediated recombination. Whole tissues were homogenized, and polyribosomes, with bound mRNAs, were immunoprecipitated with an anti-HA antibody, allowing selective enrichment of transcripts from cell types where Cre recombinase is expressed (Fig. 2B). To ensure that both the PVN and SON were included in our analysis, an ~3-mm-wide coronal section was isolated using a brain matrix (Harvard Apparatus), and the tissue was trimmed dorsal to the hypothalamus and laterally on either side of the amygdala. Sim1 was enriched approximately fivefold in immunoprecipitated mRNA from
both the hypothalamus and kidney (Fig. 2C). Avp mRNA was enriched 11-fold when immunoprecipitated mRNA was compared with total input mRNA (Fig. 2C, left), indicating that Sim1-Cre selectively targets the neurons known to produce AVP. The twofold higher enrichment of Avp mRNA compared with Sim1 mRNA was unexpected but could result if the Sim1-Cre transgene is not expressed at levels high enough to achieve recombination in all Sim1 mRNA-expressing neurons.

In the hypothalamus, Cre recombination occurred primarily in neurons since the oligodendrocyte marker 2′,3′-cyclic nucleotide 3′-phosphodiesterase was deenriched compared with input RNA (Fig. 2C, left). In the kidney, Aqp2 mRNA was enriched approximately threefold, whereas the intercalated cell marker Slc26a4 was found to deenrich from the immunoprecipitated transcripts (Fig. 2C, right). The limited expression of β-galactosidase throughout the inner medulla and renal papilla (Fig. 2A, right) was also confirmed through deenrichment of mRNA for renal urea transporter UT-A1 (Slc14a2; Fig. 2C, right). We conclude that Sim1-Cre initiates expression of RlαB in both the hypothalamic neurons responsible for synthesizing AVP and in principal cells of the renal collecting duct, where AVP acts to regulate AQP2 expression and function. Since PKA inhibition in either hypothalamic AVP neurons or in kidney AQP2-expressing epithelial cells could potentially lead to the observed diabetes insipidus (DI) phenotypes, we examined the relative contribution of neural and renal expression of RlαB in more detail.

Hypothalamic expression of RlαB does not inhibit AVP production or release. Because Avp mRNA was highly enriched in our RiboTag immunoprecipitates, we sought to determine if the DI phenotype in RlαB-ON mice was mediated through attenuated PKA activity in AVP-expressing neurons of the PVN. We used the Sim1-Cre mouse to activate RiboTag in hypothalamic neurons and used antibodies specific for AVP or RPL22-HA. As shown in Fig. 3A, AVP and RPL22-HA were coexpressed in a subset of neurons concentrated within the PVN. Many species, in particular mice, also have additional AVP-expressing neurons scattered in accessory nuclei throughout the hypothalamus, which may be important in AVP-dependent behavioral regulation (50); we identified AVP-expressing neurons that were not targeted by Sim1-Cre (Fig. 3A).

We isolated 1-mm coronal sections of the hypothalamus and took punches of the PVN to assess total kinase activity in lysates. Although RlαB is expressed in the PVN, we were unable to detect any significant decrement in PKA activity in RlαB-ON mice in the absence or presence of cAMP (Fig. 3B).

Fig. 1. Phenotypic analysis after Sim1-Cre-mediated expression of a dominant negative PKA regulatory subunit (RlαB). A: COOH-terminal exons of Prkar1a gene showing activation of the RlαB mutation (G324D) after Cre-mediated recombination. RlαB-OFF mice are heterozygous for Prkar1a in all tissues. B: breeding strategy and generation of experimental animals. C–G: average daily food intake (C), reproductive fat pad weight (D), body weight (E), average water intake per hour measured over a 5-day period (F), and urine output per hour measured over a 4-h period (in G) in control (WT) and RlαB-OFF and RlαB-ON mice (n = 5–8 mice/group). *P < 0.05; **P < 0.01; ***P < 0.001. Values were measured in 8-wk-old male and female mice and represent means ± SE.
This is not particularly surprising since we expected that the RlxB allele would only be expressed in a small subset of total cells in the punch. We then isolated total mRNA from the hypothalamus and analyzed Avp transcript levels using primers that target exon 1 of Avp mRNA. Avp mRNA was slightly elevated in RlxB-ON mice with free access to water (Fig. 3), however, there was no measurable difference among control mice and those expressing RlxB after 24-h dehydration (Fig. 3C). Although there was no observable difference in AVP peptide concentrations in urine after RlxB activation compared with control groups with free access to water (Fig. 3D), it is important to note that RlxB mice produce 2.5- to 3-fold more urine, and, therefore, daily excretion of AVP in the urine is significantly increased. The 1.5-fold increase in AVP mRNA suggests a compensatory response to nephrogenic DI that could be caused by inhibition of PKA in renal principal cells. The degree of PKA inhibition in RlxB-ON tissue is dependent on the normal level of expression of Rlx compared with expression of RIIα, RIIα, and RIIβ. Since many neurons express high levels of RIIβ, it is possible that there is not sufficient Rlx being made in AVP neurons to inhibit AVP production. Alternatively, other PKA-independent pathways are important regulators of Avp gene expression, as recently suggested (9, 20).

Inhibition of PKA lowers total AQP2 protein levels in vivo. The lack of RlxB inhibition of Avp mRNA or circulating AVP peptide levels suggested that the DI might be primarily due to renal defects in RlxB-ON mice. We crossed Sim1-Cre mice to a reporter line that expresses the fluorescent tdTomato protein after Cre-mediated recombination (36). As shown in Fig. 4A, tdTomato localizes to cells of the inner medulla that also express the principal cell-specific water channel AQP2. Expression of RlxB is sufficient to disrupt the subapical membrane localization of AQP2 (Fig. 4B), and Western blot analysis of inner medulla lysates revealed a significant decrease in total AQP2 protein (RlxB-ON: 1.02 ± 0.03 vs. RlxB-ON: 0.42 ± 0.01, P < 0.001; Fig. 4C). Interestingly, Aqp2 mRNA levels were not significantly different when total RNA from the inner medulla was analyzed by RT-PCR (RlxB-ON: 0.99 ± 0.10 vs. RlxB-ON: 0.82 ± 0.05, P > 0.05; Fig. 4D). Kinase activity assays confirmed that inner medulla RlxB expression was sufficient to inhibit PKA activity in both the absence and presence of 5 μM cAMP (Fig. 4E). Despite the decrease in kinase activity, there were no detectable changes in RIIα or RIIα subunit expression, as shown by Western blot analysis (Fig. 4F). As expected, RlxB-Off mice expressed significantly less Rlx protein due to inactivation of one Prkarla allele (Fig. 4F). Rlx protein levels were increased in the inner medulla of RlxB-ON mice, and this was expected since the Rlx subunit will bind to the C subunit even in the presence of cAMP, and we have demonstrated that Rlx is protected from degradation in the intact PKA holoenzyme (1).

RlxB expression attenuates the response to V2R activation. To assess the in vivo response of the kidney to acute V2R activation, mice expressing the activated RlxB allele (RlxB-ON) were water loaded by oral gavage to suppress circulating AVP levels. After 1 h, mice were then given an intraperitoneal...
AQP2 and Fluid Homeostasis

Injection of vehicle or the V2R agonist DDAVP (0.1 μg/kg body wt), and urine was collected for 2 h. This dose of DDAVP prevents water load-induced diuresis in WT mice (48). RlxαB-ON mice displayed significantly lower urine osmolality compared with control mice after ad libitum water consumption. However, acute water loading reduced urine osmolality to a similar level in both control and RlxαB-ON mice (Fig. 5A). DDAVP injection increased urine osmolality in all three genotypes, but urinary concentrating ability was still significantly impaired in RlxαB-ON mice (Fig. 5A). This was not due to a lack of activation of V2Rs since urinary cAMP excretion in response to acute DDAVP was not impaired in RlxαB-ON mice (Fig. 5B). Localization of AQP2 in RlxαB-ON collecting ducts was partially rescued under these conditions (Fig. 5C), and this is consistent with the increased urine osmolality seen in DDAVP-treated RlxαB-ON mice (Fig. 5A). We next examined the levels of AQP2 and phospho-AQP2 (Ser256) by Western blot analysis. Mice were water loaded and, after 1 h, received an intraperitoneal injection of vehicle or DDAVP (0.1 μg/kg). Total AQP2 protein increased by almost twofold in control and RlxαB-ON mice after only 20 min of DDAVP treatment, suggesting a rapid stabilization of AQP2 protein. AQP2 phosphorylation was also induced two- to threefold by DDAVP in control and RlxαB-ON mice (Fig. 5D). Nevertheless, total levels of AQP2 protein in RlxαB-ON kidneys remained low, explaining the deficit in urine concentrating ability. These results demonstrate that RlxαB expression interfered with AVP signaling downstream from the V2R in the kidney and led to decreased levels of AQP2 protein.

Prolonged dehydration increases total AQP2 expression in both control and RlxαB-ON mice. Long-term regulation of AQP2 protein levels is thought to depend on increased transcription of the Aqp2 gene (15). However, recent studies have implicated PKA in the regulation of AQP2 protein stability through changes in the phosphorylation of Ser256 and Ser261 (41, 43). We next determined the effect of RlxαB expression and chronic PKA inhibition on AQP2 levels after prolonged osmotic stress. We subjected control and RlxαB-ON mice to 24-h dehydration and analyzed the ability of the kidne to concentrate urine as well as the localization and expression levels of AQP2. RlxαB-ON mice displayed an improved urinary concentrating ability after 24-h dehydration (Fig. 6A), although they were still deficient compared with WT and RlxαB-OFF control mice. We isolated total RNA from control and RlxαB-ON kidneys and analyzed mRNA content for water channels involved in urinary concentration. Total transcript levels for Aqp2 and Aqp3 were not significantly different between RlxαB-OFF and RlxαB-ON mice with ad libitum water consumption and were induced twofold by dehydration (Fig. 6B). As expected, transcripts for the vasopressin renal-type receptor (Avp2r) and Aqp4 did not change with dehydration; similarly, they were not significantly different among the three genotypes (data not shown). After 24-h dehydration, AQP2 apical localization was partially restored in RlxαB-ON mice (Fig. 6C, top), whereas total AQP2 protein was still reduced under these conditions (Fig. 6C, bottom). Total AQP2 protein levels were increased approximately fivefold in WT and RlxαB-OFF kidney, suggesting that AQP2 levels are being regulated at both transcriptional and posttranscriptional levels (Fig. 6D). Total AQP2, AQP2 phosphorylation, and urinary concentrating ability all remained deficient in RlxαB-ON kidneys under dehydration conditions, despite the normal expression and induction of Aqp2 mRNA (Fig. 6D). As observed with acute activation of V2Rs, prolonged osmotic stress resulted in a significantly larger fold increase in AQP2 phosphorylation in RlxαB-ON mice, accounting for their sustained ability to concentrate their urine under these conditions.

PKA is targeted to subcellular locations through its interaction with A-kinase anchoring proteins (AKAPs), allowing for highly specific responses to unique ligands. PKA activity has been shown to copurify with AQP2-containing vesicles (31), and the kinase has been reported to colocalize or directly interact with AQP2 through its interaction with at least three different AKAPs (22, 28, 47). Since the majority of AKAPs bind RII subunits of PKA with high affinity, we examined urinary concentrating ability in mice lacking either Rlxα or RIIβ regulatory subunits (7, 13). Rlxα subunits colocalized with AQP2 in principal cells of the inner medulla (Fig. 7A) and, by Western blot analysis, we do not detect expression of RIIβ in the inner medulla (data not shown). Mice with a targeted disruption in either Rlxα or RIIβ subunits displayed no changes in urine osmolality with ad libitum water consumption, and these mice maintained their ability to concentrate urine.
their urine in response to 24-h dehydration (Fig. 7B). These results suggest that anchoring of the type II kinase is not a specific requirement for proper function of AQP2 channels.

**DISCUSSION**

In the present study, we demonstrated that partial suppression of PKA activity through RlαB expression is sufficient to disrupt fluid homeostasis and alter the localization and expression of AQP2 protein. Surprisingly, our results suggest a major posttranscriptional role for PKA in the maintenance of total AQP2 protein levels in vivo both during ad libitum water consumption and after prolonged dehydration. Sim1-Cre-mediated expression of RlαB gave rise to a striking DI phenotype, and more detailed analysis revealed that this pathology is predominantly nephrogenic rather than neurogenic in origin.

Sim1-Cre is expressed in both the inner medulla of the kidney and the PVN/SON of the hypothalamus, where AVP is synthesized and then transported to the posterior pituitary. Using both in vivo and in vitro approaches, it has been previously demonstrated that elevated serum osmolality or exposure to hyperosmotic solutions resulted in increased cAMP levels in the SON and, to a lesser extent, in the PVN (8). cAMP-dependent Avp transcriptional regulation may occur through PKA-mediated phosphorylation of CREB, which binds to putative CREs identified in the Avp promoter (27). Indeed, viral overexpression of the endogenous PKA inhibitor PKI blunted AVP induction in the PVN in response to dehydration (61), whereas Sim1-Cre-mediated disruption of CREB1 in the PVN reduced basal AVP protein levels (9). In contrast, recent work has shown that a dominant negative form of CREB reduced expression of the immediate early gene c-fos but had no effect on AVP production in vivo (35). These results suggested that Avp expression may be regulated by additional members of the ATF family of transcription factors. CREB3L1 has been recently shown to bind to the promoter region of the Avp gene and increase AVP expression in vivo (20); however, it is important to note that this transcription factor is activated by regulated proteolysis rather than PKA-mediated phosphorylation (42). Despite Sim1-Cre-driven reporter activity in the PVN (Figs. 2A and 3A), we were unable to detect any inhibition of Avp mRNA or urinary AVP peptide levels (Fig. 3, C and D) that might explain the DI phenotype.
This supports the possibility that PKA-independent regulation of the Avp gene by CREB3L1 or other transcription factors may be the dominant pathway. In RΔαB-ON mice with free access to water, Avp mRNA was increased by 1.5-fold in the RΔαB-ON hypothalamus (Fig. 3C), likely as a response to DI induced by RΔαB expression in the kidney. A threefold increase in Avp mRNA was observed in a mouse with DI caused by deletion of adenylate cyclase type 6, which is highly expressed in the kidney (49).

Sim1-Cre-dependent activation of RΔαB in the kidney leads to a dramatic inhibition of water reabsorption. AVP regulates collecting duct water permeability through both short- and long-term mechanisms. Whereas we observed no significant change in Aqp2 mRNA, there was a 60% decrease in the level of APQ2 protein in the RΔαB-ON kidney (Fig. 4C). This suggests a role for PKA in regulating the posttranslational stability of AQP2, and we hypothesize that this depends on PKA-dependent phosphorylation events that occur in the COOH-terminal tail of AQP2. PKA-mediated phosphorylation of Ser256 is well accepted as a required event for apical exocytosis of AQP2-containing vesicles. Both in vitro and in vivo, Knepper and colleagues (23) demonstrated that phospho-

Fig. 5. Effect of desmopressin (DDAVP) administration on urinary concentration and AQP2 phosphorylation and trafficking. A: mice were water loaded to suppress endogenous AVP levels and, after 1 h, injected with vehicle or DDAVP (0.1 μg/kg). Urine was collected for 2 h, and osmolality was assessed as described in EXPERIMENTAL PROCEDURES. n = 4. *P < 0.05; #P < 0.001. B: in response to DDAVP injection, urinary cAMP excretion was assessed by ELISA in control and RΔαB-ON mice (n = 3). C: control and RΔαB-ON mice were water loaded as in A and euthanized 20 min after vehicle or DDAVP injection, and AQP2 localization was assessed by immunohistochemistry. D: additional groups of mice were treated as in C, and inner medullas were microdissected and dounce homogenized in RIPA buffer for Western blot analysis. Representative Western blots and quantitation of total AQP2 and phosphorylated (P-)AQP2 (Ser256) are shown. n = 3. *P < 0.05; **P < 0.01. β-Actin was used as a loading control.
Ser256 is a priming site for subsequent phosphorylation at Ser269, a residue that regulates endocytosis of AQP2 from the apical membrane. Impaired exo- or endocytosis of AQP2 has been shown to alter the stability and half-life of the protein in AQP2-expressing cell lines. In Madin Darby canine kidney cells, loss of phosphorylation at either site has effects on the stability of AQP2 protein, as both S256A and S269A mutants exhibited reduced half-lives (41). A potentially conflicting result emerged from the genetic analysis of a mouse line with a spontaneous mutation leading to autosomal recessive congenital progressive hydronephrosis (Cph). Cph mice carried a S256L mutation that prevented phosphorylation at that site, and mice displayed severe DI symptoms in addition to Cph. The S256L AQP2 protein appeared to insert into the basolateral membranes, and there was an induction of both AQP2 mRNA and protein in kidneys from Cph mice (38). Our results showing a rapid increase in AQP2 protein after only 20 min of DDAVP stimulation in water-loaded animals are in agreement with the rapid increases in AQP2 protein observed in primary cultures of rat inner medullary collecting duct cells after either AVP or forskolin addition (43).

Phosphorylation of AQP2 alters its interactions with other proteins, including components of the actin cytoskeleton that regulate its trafficking to the apical membrane. AVP induces depolymerization of F-actin in the subapical cortex of the principal cell, a step proposed to remove the dense actin barrier...
to the trafficking and apical exocytosis of AQP2 vesicles (53). PKA-dependent phosphorylation of AQP2 at Ser\textsuperscript{256} decreases its interaction with G-actin while producing a high-affinity binding site for tropomyosin-5b (TM5b) (45). Binding to phospho-AQP2 (Ser\textsuperscript{256}) reduced TM5b interactions with F-actin, thereby enhancing its depolymerization and facilitating movement of AQP2 vesicles to the apical membrane. A direct effect of RI\(\alpha\)B expression on actin polymerization cannot be ruled out as PKA phosphorylates RhoA, causing dissociation from membrane-bound proteins, including Rho/Rac/Cdc-42-activated kinases, independent of GTP/GDP binding (59). Similar to the interaction between phospho-AQP2 and TM5b, PKA-mediated inhibition of RhoA would favor depolymerization of F-actin. Indeed, inhibition of RhoA with Clostridium botulinum toxin C3 increased apical accumulation of AQP2 in CD8 collecting duct cells in the absence of V2R activation (58). The delocalization of AQP2 in RI\(\alpha\)B-ON mice under ad libitum water consumption conditions that we observed in this study may reflect reduced actin depolymerization, through direct or indirect actions, and impairment of normal trafficking of AQP2 vesicles.

While PKA-dependent phosphorylation has been clearly implicated in the short-term regulation of AQP2, its role in long-term AQP2 regulation is not well understood, particularly in vivo. PKA has been shown to regulate AQP2 expression through phosphorylation of CREB and increased transcriptional activity of the Aqp2 promoter region (26, 37, 64). However, our results show that Aqp2 mRNA levels remained normal in RI\(\alpha\)B-ON mice either during free access to water (Figs. 4\(D\) and 6\(B\)) or after 24-h dehydration (Fig. 6\(B\)), demonstrating that Aqp2 mRNA levels are not affected by expression of the dominant negative PKA mutant in vivo. Alternative pathways have been suggested as regulators of the Aqp2 gene. Hyperosmotic culture medium is sufficient to maintain AQP2 expression in isolated, cultured collecting ducts, whereas a dominant negative form of tonicity element-binding protein [TonEBP or nuclear factor of activated T cells 5 (NFAT5)] decreased Aqp2 mRNA levels in mice with free access to water (30, 57). Surprisingly, dominant negative TonEBP mice displayed normal Aqp2 mRNA levels when challenged with dehydration. These data suggest that Aqp2 gene transcription can be regulated by AVP-dependent but cAMP- and PKA-independent mechanisms in the dehydrated state. AVP treatment also increased intracellular Ca\textsuperscript{2+} levels in isolated medullary collecting ducts (10), and activation of calcineurin and NFATc1 increased Aqp2 mRNA levels in a collecting duct cell line (34). Further studies will be needed to address the in vivo role of Ca\textsuperscript{2+}-dependent Aqp2 transcriptional regulation in response to dehydration.

Treatments that bypass inactive V2Rs in patients with X-linked nephrogenic DI would be an attractive therapeutic option (3). Activation of alternative G\(\alpha\)-linked G protein-coupled receptors expressed on inner medullary collecting duct cells with ligands like calcitonin (4), glucagon (63), or ONO, a selective ligand for the EP4 subtype of the PGE\(_2\) receptor (33), increased cAMP levels in the inner medullary collecting duct and improved water reabsorption. ONO was used to treat mice with V2R knockout induced in adulthood, and this significantly improved the DI phenotype and produced a modest increase in AQP2 protein (33). However, none of these agonists have been shown to induce Aqp2 mRNA and may be acting primarily through short-term regulation of AQP2 phosphorylation and membrane localization. Interestingly, genetic disruption of adenylate cyclase type 6 virtually ablated the AVP-induced increase in cAMP levels in the inner medulla but had no effect on the basal level of AQP2 protein (49). In contrast, the DI +/+ mouse (18, 24), which has constitutively high type IV phosphodiesterase activity and impaired cAMP accumulation in the collecting duct, displays a drastic reduction in both AQP2 mRNA and protein. It will be essential to determine the ability of drugs that can bypass the V2R to impact short-term AQP2 phosphorylation and membrane insertion, AQP2 protein stability, and the long-term transcriptional regulation of Aqp2 in the treatment of X-linked nephrogenic DI.

The PKA holoenzyme is targeted to distinct subcellular locations through its interactions with AKAPs, molecular scaffolds that serve to generate signaling specificity in response to increased intracellular cAMP levels. AKAP-mediated targeting of PKA activity is required for AQP2 translocation to the apical surface in cultured primary principal cells, and AKAP76 (also known as AKAP188) was first cloned from the rat kidney and copurified with AQP2-containing vesicles (22, 29). While the RI\(\alpha\) regulatory subunit is not required for normal urinary concentration in mice subjected to dehydration (Fig. 7\(B\)), these data do not preclude a role for targeted PKA activity in regulating AQP2 translocation to the apical membrane. We have previously demonstrated robust posttranslational stabilization of the RI\(\alpha\) subunit in both RI\(\alpha\) and RI\(\beta\) knockout mice (7, 13); PKA is likely still targeted to AQP2-containing vesicles through interactions of the RI holoenzyme with AKAP7 and/or AKAP11, both of which bind to the RI\(\alpha\) subunit (6, 14). It is interesting to speculate that our present findings, combined with the studies outlined above, point to an even more intricate compartmentalization of cAMP and PKA than is currently
appreciated with regard to AQP2 expression and trafficking in the kidney.

Our results suggest a robust posttranscriptional role for PKA in regulating whole cell AQP2 levels in the collecting duct under both normal conditions with water freely available and after prolonged dehydration. The in vivo induction of Aqp2 mRNA in response to dehydration was only twofold, and the partial inhibition of PKA by expression of RIIβ was not sufficient to block this Aqp2 mRNA induction. However, levels of AQP2 protein were reduced dramatically in both control and dehydrated animals. Phosphorylation of AQP2 in the RIIβ-ON kidney is also inhibited, although the link between AQP2 phosphorylation and protein turnover remains unclear. Thus, the RIIβ-ON mouse represents a novel model of nephrogenic DI that may help dissect the role of PKA in the regulation of water excretion.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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