Long Term Regulation of Aquaporin-2 Expression in Vasopressin-responsive Renal Collecting Duct Principal Cells*

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Kidneys are the major determinant of body water and electrolyte composition. Water reabsorption across the membranes of renal epithelial cells occurs through a complex process. Approximately 70 and 15% of the glomerular filtrate is reabsorbed in the proximal tubule and thin descending limb of Henle’s loop, respectively. In contrast, the ascending limb of Henle’s loop and the distal convoluted tube are impermeable to water. These segments empty into the collecting duct (CD), a chief site where tight regulation of water reabsorption occurs. In this segment, and in the connecting tubule of some species as well (1, 2), the excretion of electrolyte-free water is adjusted by principal cells under the control of the antidiuretic hormone [8-arginine]vasopressin (AVP) (3).

Water movement across renal epithelial cells is facilitated by the presence of water channels of the aquaporin (AQP) protein family. Aquaporins exhibit a conserved homotetrmeric organization, and the expression of different members of the aquaporin family is tissue-specific. AQP1 accounts for the transcellular selectivity of body water reabsorption across renal epithelial cells where AVP regulates water reabsorption across the principal cell epithelium. AQP2 is located in subapical intracellular vesicles and in the apical plasma membrane (7, 8), whereas AQP3 and AQP4 are both located in the basolateral membrane (9, 10). Of all aquaporins, AQP2 is the principal target of AVP. Acute increases in plasma AVP concentration leads to translocation of AQP2 from intracellular storage vesicles to the apical plasma membrane and results in increased CD water permeability (11). Sustained increases in plasma AVP levels raise expression levels of both AQP2 and AQP3 allowing maximal water permeability across renal CD (12). AVP exerts its effect by binding to V2 receptors located in the basolateral membrane of CD principal cells and inner medullary collecting duct cells, resulting in activation of the Gs/adenylyl cyclase system (13), increased intracellular cAMP concentration, and cAMP-dependent protein kinase activation. Mutations in either AQP2 or the V2 receptor are responsible for reduced expression levels of AQP2 of inherited forms of nephrogenic diabetes insipidus (14, 15).

Several studies have documented the long term control of AQP2 expression by AVP (16, 17, 20, 34). Prolonged infusion with AVP has been shown to increase the water permeability of renal CD in AVP-deficient Brattleboro rats, an effect that correlates well with increased levels of AQP2 mRNA and protein (16). Similarly, normal rats infused with AVP develop hyponatremia and exhibit increased AQP2 expression levels (17). AQP2 expression can also be increased under particular circumstances such as cardiac failure (18, 19), liver cirrhosis (20), and pregnancy (21) where a hyponatremic state is associated with non-osmotic AVP release. These findings together with the identification of cAMP-responsive elements in the 5′-flanking region of the AQP2 gene (22) suggest that AVP increases the transcription rate of the AQP2 gene.

Fine regulation of water reabsorption by the antidiuretic hormone [8-arginine]vasopressin (AVP) occurs in principal cells of the collecting duct and is largely dependent on regulation of the aquaporin-2 (AQP2) water channel. AVP-inducible long term AQP2 expression was investigated in immortalized mouse cortical collecting duct principal cells. Combined RNase protection assay, Western blot, and immunofluorescence analyses revealed that physiological concentrations of AVP added to the basal side, but not to the apical side, of cells grown on filters induced both AQP2 mRNA and apical protein expression. The stimulatory effect of AVP on AQP2 expression followed a V2 receptor-dependent pathway because [deamino-8-d-arginine]vasopressin (dDAVP), a specific V2 receptor agonist, produced the same effect as AVP, whereas the V2 antagonist SR121463B antagonized action of both AVP and dDAVP. Moreover, forskolin and cyclic 8-bromo-AMP fully reproduced the effects of AVP on AQP2 expression. Analysis of protein degradation pathways showed that inhibition of proteasomal activity prevented synthesis of AVP-inducible AQP2 mRNA and protein. Once synthesized, AQP2 protein was quickly degraded, a process that involves both the proteasomal and lysosomal pathways. This is the first study that delineates induction and degradation mechanisms of AQP2 endogenously expressed by a renal collecting duct principal cell line.

Kidneys are the major determinant of body water and electrolyte composition. Water reabsorption across the membranes of renal epithelial cells occurs through a complex process. Approximately 70 and 15% of the glomerular filtrate is reabsorbed in the proximal tubule and thin descending limb of Henle’s loop, respectively. In contrast, the ascending limb of Henle’s loop and the distal convoluted tube are impermeable to water. These segments empty into the collecting duct (CD), a chief site where tight regulation of water reabsorption occurs. In this segment, and in the connecting tubule of some species as well (1, 2), the excretion of electrolyte-free water is adjusted by principal cells under the control of the antidiuretic hormone [8-arginine]vasopressin (AVP) (3).

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The abbreviations used are: CD, collecting duct; AQP, aquaporin; AVP, [8-arginine]vasopressin; dDAVP, [1-deamino,8-d-arginine]vaso-
Cultured kidney epithelial cells transfected with AQP2 cDNA have proven to be a valuable tool for the study of short term action of AVP on AQP2 expression (23, 24). However, they cannot be used for long term regulation studies that require intact cis- and trans-acting DNA domains. Attempts to characterize long term AQP2 expression in primary cultures of kidney epithelial cells have been hampered by the rapid down-regulation of endogenously expressed AQP2 (25), possibly because of the presence of negatively acting cis-elements present in the AQP2 gene (26). We have established previously a novel immortalized clonal collecting duct cell line, mpkCCDc14, which was derived from microdissected cortical collecting ducts of an SVJP/Tag transgenic mouse (27). These mpkCCDc14 cells exhibit many major functional properties of CD principal cells including electrogenic NaCl transport stimulated by aldosterone and AVP (27, 28). The high differentiation state of these cells, which develop into tight epithelium, has been exploited for analysis of regulated NaCl transport mediated by the apical amiloride-sensitive NaCl channel (27, 29, 30) and basolateral Na+-K+-ATPase (31, 32) and has allowed identification of a panel of genes that are stimulated or repressed by aldosterone and vasopressin (33).

In this study, we show that mpkCCDc14 cells grown on permeable filters maintain AVP-inducible AQP2 expression and can be used as a cellular model to analyze the mechanisms that govern the long term effect of AVP on AQP2 expression. These cells produce large amounts of both AQP2 mRNA and protein in response to physiological concentrations of AVP. Synthesis of AQP2 mRNA was found to be dependent on the proteolytic activity of the proteasome. In addition, newly synthesized AQP2 protein was found to have a short half-life, and both proteasomal and lysosomal proteolytic pathways were found to participate in its degradation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Mouse mpkCCDc14 cells grown in modified DM medium (Dulbecco’s modified Eagle’s medium/Ham’s F-12, 1:1 v/v; 60 mM sodium selenite; 5 μM transferrin; 2 μM glutamine; 50 nM dexamethasone; 1 nM triiodothyronine; 10 μg/ml insulin; 4 μg/ml aprotinin; 1% Triton X-100; pH 7.4) (27) were used. All experiments were performed on confluent cells seeded on permeable filters (Transwell polycarbonate filters, 0.4-μm pore size). Cells were grown in DM medium until confluent (day 6 after seeding) and then in DM containing no epidermal growth factor, hormones, or fetal calf serum for an additional 24 h before use. The medium was changed every 2 days, and all experiments were performed between the 20th and 35th passages.

**Protein Extraction**—Strips of rat kidney cortex were excised and frozen in liquid nitrogen, ground to powder, and homogenized in 500 μl of ice-cold lysis buffer (20 mm Tris-HCl; 2 μM EDTA; 2 μM EGTA; 30 μM NaF; 50 mM Na2HPO4; 2 μM Na3VO4; 1 mM 4-(2-ami-noethyl)benzenesulfonyl fluoride; 10 μg/ml leupeptin; 4 μg/ml aprotinin; 1% Triton X-100; pH 7.4). After incubation in the absence or presence of AVP and/or drugs, confluent cultured mpkCCDc14 cells grown on filters or flasks were rinsed twice with phosphate-buffered saline and then homogenized in 150 μl of ice-cold lysis buffer (see above). Protein concentrations were measured by the BCA protein assay (Pierce).

**Western Blot Analysis**—Equal amounts of protein samples were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). Membranes were blocked by incubation with Tris-buffered saline (20 μM Tris, 150 μM NaCl) containing 0.2% (w/v) Nonidet P-40 and 5% (w/v) nonfat dry milk for 30 min at room temperature. Membranes were probed overnight at 4 °C with a polyclonal rabbit anti-AQP2 antibody (1:20,000) (18) and then with secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (1:20,000) (Transduction Laboratories, Lexington, KY) for 1 h at room temperature. The membranes were washed three times with Tris-buffered saline containing 0.2% (v/v) Nonidet P-40, and the antigen-antibody complexes were detected by the Super Signal Substrate method (Pierce). Identified protein bands were quantified using a video densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**Immunofluorescence Studies**—Confluent mpkCCDc14 cells grown on filters were incubated in a serum- and hormone-deprived medium supplemented or not with 10−8 M AVP for 24 h. Cells were fixed with 2% paraformaldehyde for 20 min at room temperature, permeabilized with 0.25% (v/v) triton (Nonidet P-40) for 10 min, and then rinsed with phosphate-buffered saline. Fixed cells were permeabilized with 0.5% Triton X-100 and incubated with the rabbit polyclonal anti-rat AQP2 antibody (dilution 1:50) for 1 h at room temperature. After three rinses with phosphate-buffered saline, specimens were incubated with a Cy3-conjugated goat anti-rabbit IgG antibody (dilution 1:100) (Jackson ImmunoResearch. Laboratories Inc., West Grove, PA) for 30 min at room temperature. Nuclei were counterstained with Sytox (dilution 1:50) (Molecular Probes, Eugene, OR). Specimens examined by confocal laser scanning microscopy (Leica, Wetzlar, Germany) were viewed in the x-y and x-z planes, and the images were photographed.

**RNase Protection Assay**—Mouse genomic DNA was extracted from mpkCCDc14 cells using the RNaseasy Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. A mouse AQP2 cDNA (NCBI accession number NM_009699) fragment coding for the 5′-719-nucleotide sequence was PCR-amplified using sense and antisense primers containing EcoRI and XbaI restriction sites, respectively, and cloned into pCIneo (Promega, Madison, WI). Computer-assisted alignment sequence analyses confirmed the specificity of the cDNA fragment used. The sequence of the PCR-amplified fragment was checked by sequencing the plasmid. Hybridization was then linearized with XbaI restriction enzyme, and 1 μg was used to produce antisense AQP2 transcripts (riboprobes) using T3 RNA polymerase in the presence of 50 μCi of [α-32P]UTP (Amersham Biosciences). A linearized pT7 RNA 18 S plasmid (Ambion, Austin, TX) was used to produce an 18 S rRNA antisense probe, used as an internal standard, which was transcribed with T7 RNA polymerase in the presence of 5 μCi of [α-32P]UTP to avoid signal saturation due to the greater abundance of 18 S rRNA as compared with AQP2 rRNA. Total RNA was extracted from cultured cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Four μg of riboprobe and 21 μg of yeast tRNA were used for hybridization with 2 × 105 cpm AQP2 probe and 5 × 105 cpm 18 S rRNA probe. Yeast tRNA (25 μg) was used as a negative control. Hybridization was performed for 60 min at 70 °C followed by RNase A/T1 mixture digestion (Ambion, Austin, TX) for 30 min at 37 °C. The reaction was terminated by the addition of SDS and proteinase K. RNA duplexes were extracted with phenol/chloroform/isoamyl alcohol and precipitated with ammonium acetate/ethanol. Samples were then denatured in gel loading buffer at 95°C for 5 min, run together with non-digested riboprobes on a 6% polyacrylamide sequencing gel, and autoradiographed. Identified fragments were quantified using a video densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**RESULTS**

**Endogenous AQP2 Expression of Untreated and AVP-treated Mouse Collecting Duct Principal Cells**—The expression of AQP2 mRNA was analyzed by RNase protection assay (RPA) in confluent mpkCCDc14 cells. Cells grown on filters were incubated 9 h without or with 10−8 m AVP and in the absence or presence of 2×10−8 m actinomycin D. All agents were added to the basal medium. Protected fragments of expected size for the 18 S rRNA probe were visible in each tested condition at similar intensities (Fig. 1A, bottom panel) confirming that equal amounts of RNA were loaded to each lane. In untreated cells, a signal corresponding to protected fragments of the AQP2 mRNA probe could be detected but only after very long exposure times, indicating very low basal AQP2 mRNA expression levels (Fig. 1A, lane 1, top panel). The presence of AQP2 mRNA in untreated cells was nevertheless confirmed by reverse transcription-PCR analyses using specific primers for AQP2 (data not shown). Importantly, cells incubated 9 h in the presence of 10−8 m AVP exhibited dramatically increased expression levels of AQP2 mRNA (Fig. 1A, lane 2, top panel). An additional faint band of about 31 kDa that was endoglycosidase H-sensitive (data not shown) was
provided 9 h with 10⁻¹⁰ M AVP, and AQP2 protein expression sharply increased when greater concentrations of AVP were added to the cell medium. Half-maximal and maximal induction were achieved at 10⁻¹⁰ and 10⁻⁹ M AVP, respectively. AVP-induced AQP2 expression was also found to be time-dependent (Fig. 2, C and D). AQP2 protein was first detected 3 h after addition of 10⁻⁹ M AVP to the basal medium and gradually increased thereafter. For incubation times exceeding 24 h, the amount of AQP2 protein continued to increase, and by 72 h AQP2 protein expression was 2-fold greater than that observed after 24 h of AVP treatment (data not shown).

In agreement with Western blot results (Fig. 1B), immunofluorescence analysis of untreated confluent mpkCCDC₁₄₄ cells grown on filters revealed almost no fluorescent signal (Fig. 3A, panel A), whereas cells incubated 24 h with 10⁻⁸ M AVP added to the basal medium exhibited intense AQP2 immunostaining (Fig. 3A, panel B). Confocal laser scanning microscopy analysis of x-z planes revealed that most AQP2 immunostaining was detected at the apical pole of AVP-treated cells, whereas the basolateral pole remained unaltered (Fig. 3A, panel D).

The observations made by confocal laser scanning microscopy suggest that the predominant apical localization of the AQP2 protein is conserved in mpkCCDC₁₄₄ cells. We next determined whether these cells also retain a polarized expression of AVP receptors. Although a strong AQP2 protein signal was detected by Western blotting when 10⁻⁹ M AVP was applied for 24 h to the basal medium (Fig. 3B, lane 3), no specific bands corresponding to AQP2 were detected when cells were grown in the absence of AVP (lane 1), when AVP was added to the apical medium for 24 h (lane 2), or when cells were grown to confluence on a solid plastic support in the presence of 10⁻³ M AVP for the same period (lane 4). These results indicate that AVP receptors are predominantly located in the basolateral membrane of mpkCCDC₁₄₄ cells.

In kidney collecting duct cells, AVP controls water permeability and AQP2 expression levels by binding to V₂ receptors (34), which causes an increase in cellular cAMP content (35). We next determined whether AVP-induced AQP2 expression observed in mpkCCDC₁₄₄ cells relies on AVP binding to basolateral V₂ receptors. The strong AQP2 signal induced by incubating cells 9 h with 10⁻⁹ M basal AVP was attenuated in the presence of 10⁻⁸ M SR121463B (a generous gift of Dr. C. Serradell-Le Gal, Sanofi Research, Toulouse, France), a non-peptidic, competitive, and specific V₂ receptor antagonist (36) (Fig. 3C, lanes 2 and 3). On the other hand, basal addition of 10⁻³ M 1-desamino-8-D-arginine vasopressin (dDAVP), a preferential V₂ receptor agonist, increased AQP2 cellular content as efficiently as 10⁻⁹ M AVP (Fig. 3C, lane 4). Similar to AVP-induced AQP2 expression, AQP2 expression induced by dDAVP was attenuated by simultaneous basal addition of SR121463B (lane 5). To establish further the involvement of V₂ receptors in AVP-induced AQP2 expression, we assessed whether cAMP was able to mimic the AVP- or dDAVP-dependent increase of cellular AQP2 protein content by incubating cells 24 h with 5⁻¹⁰⁻⁶ M forskolin or with 10⁻³ 8-bromo-cyclic AMP (Fig. 3D). Compared with AVP alone, both of these compounds induced a similar rise in AQP2 protein levels (compare lane 4 to lanes 2 and 3), indicating that the action of AVP in mpkCCDC₁₄₄ cells is mediated by cAMP. These results demonstrate, as observed in vivo, that AQP2 protein expression induced by AVP or dDAVP occurs via the occupancy of functional V₂ receptors located in the basolateral membranes of mpkCCDC₁₄₄ cells.

**Role of Proteasomal and Lysosomal Proteolytic Pathways in the Control of AQP2 Expression in Mouse Collecting Duct Prin-
Principal Cells—Integral membrane proteins are mostly degraded through the lysosomal proteolytic pathway, but recent experimental evidence indicates that a subset of these proteins can also be degraded by the proteasome (37). The results presented so far strongly suggest that AVP increases synthesis of the AQP2 protein by increasing AQP2 mRNA levels. The expression levels of cellular proteins, however, depend on protein synthesis and degradation rates. We therefore investigated the degradation pathways of AQP2 protein and their role in the control of AQP2 expression levels. The respective contribution of the proteasome and the lysosome in AQP2 degradation in mpkCCD14 cells was analyzed by treating cells with specific inhibitors for each degradation pathway. Proteasomal activity was inhibited by adding 10^{-6} M lactacystin or 10^{-5} M MG132, two potent and specific inhibitors of the proteasomal pathway, to the cell medium. To analyze the contribution of the lysosome in AQP2 degradation, we used either 2x10^{-6} M leupeptin, an inhibitor of cysteine proteases, or either 10^{-7} M chloroquine or 10^{-5} M methylamine, two weak bases that increase lysosomal pH and thereby inhibit the proteolytic activity of lysosomal enzymes. We first checked that these drugs had no cytotoxic effects by measuring the transepithelial electrical resistance of confluent mpkCCD14 cells grown on filters (data not shown). None of the proteasomal or lysosomal inhibitors increased AQP2 expression when applied to the cell medium for 9-24 h in the absence of AVP, as revealed by Western blot analysis (data not shown). These results indicate that the very low AQP2 expression levels of cells grown in the absence of AVP are not due to an immediate degradation of constitutively synthesized AQP2 protein.

The effects produced by the presence of proteasomal inhibitors on AQP2 expression led to contrasting results. Indeed, when cells were simultaneously incubated 9 h with both 10^{-6} M lactacystin and 10^{-9} M AVP, the AVP-induced expression of AQP2 protein was almost completely abolished (Fig. 4A, compare lanes 2 and 3). Identical results were obtained with MG132, another structurally unrelated proteasome inhibitor (data not shown). To determine whether inhibition of proteasomal activity decreased AVP-induced AQP2 expression through a transcriptional or a translational mechanism, we measured the effect of lactacystin on AQP2 mRNA expression. Results from RPA analysis performed on the same batch of cells used for Western blot analysis clearly show that AQP2 mRNA expression induced by AVP was nearly abolished by lactacystin (Fig. 4B, compare lanes 2 and 3). Taken together, these results reveal that the increased levels of AQP2 mRNA expression in response to AVP require the functional integrity of the proteasome.

We investigated the role of the proteasome in AQP2 degradation by first incubating mpkCCD14 cells 24 h with 10^{-9} M AVP alone and then for an additional 9 h in the presence of 10^{-9} M AVP added to the cell medium without or with 10^{-6} M lactacystin. Results from Western blots performed under these experimental conditions showed that lactacystin considerably increased the amount of total AQP2 protein as compared with cells treated with AVP alone (Fig. 5, compare lanes 1 and 2, and see Fig. 5B). We further examined the effect of lactacystin on AQP2 degradation by first incubating cells 24 h with 10^{-9} M AVP alone and then for an additional 9 h in the absence of AVP (AVP chase) and in the absence or presence of 10^{-6} M lactacystin. Under these conditions, the presence of lactacystin significantly increased cellular AQP2 protein content as compared with AVP-pretreated cells incubated in the absence of both AVP and lactacystin (Fig. 5A, compare lanes 3 and 4, and see Fig. 5B). The present results show that the high levels of AQP2 protein expression observed after 24 h of AVP incubation were greatly reduced 9 h after AVP was removed from the cell medium, suggesting that AQP2 protein is quickly degraded in the absence of AVP. The observation that lactacystin reduced AQP2 degradation to a similar extent both in the absence and presence of AVP suggests that AQP2 degradation occurs re-
regardless of the absence or presence of AVP and that this process is at least partially mediated by proteasomal activity.

Integral membrane proteins are typically degraded by lysosomal proteases. We examined lysosomal AQP2 degradation in mpkCCD<sub>14</sub> cells by incubating cells 9 h with 10<sup>-9</sup> M AVP in order to induce large AQP2 protein content as compared with AVP-pretreated cells incubated in the absence of both AVP and a lysosomal inhibitor (9-h chase, Fig. 6C) in the absence (lanes 1 and 4) or presence (lanes 2 and 3) of 10<sup>-9</sup> M AVP and either without (lanes 1 and 2) or with (lanes 3 and 4) 10<sup>-6</sup> M lactacystin prior to RNA and protein extraction. A, Western blotting was performed on protein extracts (40 µg) using a polyclonal anti-AQP2 antibody. B, RPA was performed with riboprobes for AQP2 mRNA (top panel) and for 18 S rRNA (bottom panel) on 4 µg of total RNA extracted from the same batch of cells used for Western blot analysis. One of two (for RPA) or four (for Western blot) similar experiments is shown.

The results of the present study suggest that newly synthesized AQP2 protein is quickly degraded in mpkCCD<sub>14</sub> cells. AQP2 protein degradation was further analyzed by first pre-treating cells 24 h with 10<sup>-9</sup> M AVP in order to induce large expression levels of AQP2 mRNA and protein and then by incubating cells for an additional 24 h in the absence of AVP. RPA and Western blot analysis revealed that AQP2 mRNA and protein expression levels both returned to near baseline levels 24 h after AVP deprivation (Fig. 7, A and B, lanes 2 and 3). Time-dependent AQP2 degradation (Fig. 7, C and D) was next analyzed on protein extracts of cells stimulated 24 h with 10<sup>-9</sup> M AVP and then subjected to various lengths of time in the absence of AVP (chase period). Western blot analysis revealed that 1 h after AVP removal, AQP2 protein expression was found to be about 50% greater than that observed in cells...
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stimulated 24 h with AVP. This rather unexpected increase in AQP2 expression was maintained for the next 2 h. At later chase times, cellular AQP2 protein content gradually decreased and attained near base-line levels 24 h after AVP removal. Extrapolation of the AQP2 degradation curve suggests that the half-life of total AQP2 protein, obtained after 24 h of AVP incubation, is −6 h in mpkCCDcl4 cells.

DISCUSSION

The immortalized mpkCCDcl4 cells used in this study represent, to our knowledge, the first mammalian model of cortical collecting duct cells that maintain high endogenous AQP2 expression levels induced by AVP administered at physiological concentrations. We have taken advantage of this remarkable property by investigating long-term AVP-induced AQP2 expression, and several novel aspects have been revealed. Our results indicate that AVP-inducible long-term AQP2 expression relies on a cAMP-dependent increase of AQP2 mRNA which itself depends on proteasomal activity. Our results further indicate that the proteasomal and lysosomal pathways both participate in AQP2 protein degradation occurring soon after its synthesis.

A large amount of work has been done to unravel the mechanisms involved in AVP-induced targeting of AQP2 from intracellular vesicles to the plasma membrane (3). The long-term influence of AVP on AQP2 expression, however, remains largely unexplored because of the absence of an authentic cell line expressing significant amounts of endogenous AQP2 protein. The mpkCCDcl4 cell line (27) produces sufficient amounts of endogenous AQP2 protein to allow its detection by Western blot. Although very small amounts of AQP2 protein were occasionally observed in untreated cells, exogenous AVP treatment administered at physiological concentrations (i.e. at 10−10 M) greatly increased AQP2 expression levels. This effect was observed in a hormone- and serum-free medium indicating that AVP is necessary and sufficient to induce significant AQP2 expression in mpkCCDcl4 cells. AVP-induced AQP2 expression was only observed when AVP was administered to the basal medium of cells grown on porous filters. In contrast, the addition of AVP to the apical medium of cells grown on filters or to cells grown on a solid support did not increase AQP2 expression levels. These results indicate that mpkCCDcl4 cells grown on filters retain a polarized basolateral expression of AVP V2 receptors characteristic of native principal cells of the collecting duct (38). Several pieces of evidence indicate that the AVP response in mpkCCDcl4 cells is mediated by V2 receptors. First, dDAVP, a preferential V2 receptor agonist, induced AQP2 expression at the same magnitude as AVP. Second, SR121463B, a specific V2 receptor antagonist (36), decreased both AVP- and dDAVP-induced expression of AQP2. Finally, forskolin, a direct activator of adenyl cyclase, and 8-bromo-cyclic AMP, a cell-permeant analog of cAMP, fully reproduced the effect of AVP indicating that cAMP generation induces AQP2 expression via V2 receptor activation, as demonstrated previously (25) in primary cultures of rat inner medullary collecting duct cells. Together with transepithelial Na+ transport responsiveness to mineralocorticoids (27, 32) and AVP (28, 31) mediated by the amiloride-sensitive Na+ channel (ENaC) and Na+−K+−ATPase, the demonstration that these cells also exhibit AVP-induced AQP2 expression indicates that mpkCCDcl4 cells retain most of the fundamental properties of principal cells of the collecting
results from increased AVP-induced AQP2 protein expression but also AQP2 mRNA expression, it appears likely that inhibition of proteasomal activity alters the regulation of AQP2 gene transcription. On the other hand, we cannot exclude the possibility that proteasomal inhibitors decrease AQP2 mRNA stability when added to the cell medium together with AVP. Tight control of signal transduction often involves rapid degradation of transcription factors mediated by the ubiquitin-proteasomal pathway. Examples of such transcription factors include the tumor suppressor proteins p53, c-Jun, and E2f-1 (42). The IκB family of inhibitory molecules represents a good example of negatively regulated transcription factors. IκBα binds to NF-κB nuclear factors (NF-κB) and are degraded by the proteasome pathway in response to extracellular stimuli such as cytokines and stress allowing translocation of NF-κB into the nucleus (43). In the case of AQP2, a negatively acting transcription factor may bind to one or several of the negatively acting cis-elements proposed to repress AQP2 gene transcription in IMCD cells (26). The results of the present study may therefore be explained by the AVP-induced degradation, via the proteasome, of one or several transcription factors that negatively act on AQP2 expression, either by impeding the translocation of a functionally active element into the nucleus or by binding directly to the AQP2 gene. In either case, the presence of a proteasome inhibitor would block their degradation and consequently repress AQP2 expression. Further work is needed to identify the regulatory factors that govern AQP2 gene transcription in response to AVP.

Cells degrade proteins through two major systems, the lysosome and the proteasome. Lysosomes are involved in the degradation of extracellular and cytosolic molecules as well as transmembrane transporters and receptors (44), whereas the proteasome degrades cytosolic, nuclear, and membrane proteins (42) and eliminates misfolded proteins and misassembled oligomeric protein complexes at the level of the endoplasmic reticulum (45). Both degradation systems are used in renal epithelial cells as illustrated by the lysosomal degradation of the co-transporter NaPi-2, expressed in the apical membrane of proximal tubule cells (46), and by ubiquitin-proteasomal degradation of ENaC (47, 48), expressed in the apical membrane of collecting duct principal cells (40, 49). The results of the present study show that both pathways participate in the degradation of AQP2 protein in mpkCCCD14 cells. Indeed, the presence of either proteasomal or lysosomal inhibitors added to the medium of cells previously stimulated with AVP further increased AQP2 protein expression levels. It is possible that part of the AQP2 fraction protected from proteasomal degradation consists of a misfolded or aggregated AQP2 population residing in the endoplasmic reticulum. Nevertheless, the observation that fully glycosylated AQP2 is also protected from degradation by the presence of proteasomal inhibitors suggests that much of the proteasomal degradation of mature AQP2 complexes occurs outside of the endoplasmic reticulum. The similar increase in AQP2 expression in the presence of proteasome or lysosome inhibitors suggests that both degradation pathways participate equally in AQP2 degradation. Inhibition of either pathway has been observed to impede degradation of several mammalian cell membrane proteins including the platelet-derived growth factor receptor β (PDGFR-β) (41, 42). The PDGFR-β and all other class III receptor tyrosine kinases (RTKs) are degraded by the proteasome, whereas the class I RTKs are degraded by the lysosome (43). The observation that AQP2 is degraded by both pathways is therefore consistent with the functional redundancy of these pathways for the degradation of AQP2 in mpkCCD14 cells.
factor receptor (50) and the Met tyrosine kinase receptor (51). AQ2P degradation in mpkCD44 cells may be mediated by both pathways independently of each other or acting sequentially, as suggested for the degradation of connexin-43 in embryonic rat heart BWEM cells (52). Alternatively, the degradation of a protein other than AQ2P by one degradation pathway might be required for efficient AQ2P degradation by the other pathway.

The present study clearly shows that AQ2P is quickly degraded in mpkCD44 cells. This is supported by the observation that, after 9 h, AQ2P expression doubled in the presence of AVP, suggesting that AVP influences AQ2P. Moreover, AVP-chase experiments indicate that AQ2P half-life is ~6 h. The amount of AQ2P that accumulated in the presence of various degradation inhibitors was similar in the presence and absence of AVP, suggesting that AVP influences neither the degradation rate of AQ2P nor the respective contribution of the proteasome and lysosome degradation pathways.

In the light of these results, the role of AVP in long term AQ2P expression may be limited to the induction of AQ2P synthesis through the degradation, via the proteasome pathway, of negatively acting transcription factor(s). The newly synthesized AQ2P protein would then be subject to rapid degradation via the lysosomal and proteasomal degradation pathways. A short half-life, i.e. <4 h, mediated by the proteasome pathway was also found for AQ1P expressed in BALB/c fibroblasts (53). The results of the present study suggest that, along with AVP-regulated cell surface expression of AQ2P, rapid degradation of AQ2P mediated by the lysosome and proteasome pathways contribute to controlled AQ2P expression.

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Long Term Regulation of Aquaporin-2 Expression in Vasopressin-responsive Renal Collecting Duct Principal Cells

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