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Calpain-mediated AQP2 proteolysis in inner medullary collecting duct

Dechu P. Puliyanda,[a,b] Donald T. Ward,[a,c,*] Michelle A. Baum,[a] Timothy G. Hammond,[d] and H. William Harris Jr.[a,e,f]

[a] *Division of Nephrology, Children's Hospital, Harvard Medical School, Boston, MA 02115, USA*
[b] *Division of Pediatric Nephrology, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA*
[c] *School of Biological Sciences, University of Manchester, Stopford Building, Oxford Road, Manchester M13 9PT, UK*
[d] *Tulane University Medical Center and VA Medical Center, New Orleans, LA 70112, USA*
[e] *Maine Medical Center Research Institute, Portland, ME 04102, USA*
[f] *MariCal Inc., Portland, ME 04101, USA*

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Abstract

Vitamin D-elicited hypercalcemia/hypercalciuria is associated with polyuria in humans and in animal models. In rats, dihydrotachysterol (DHT) induces AQP2 water channel downregulation despite unaltered AQP2 mRNA expression and thus we investigated the mechanism of AQP2 degradation. Incubation of AQP2-containing inner medullary collecting duct (IMCD) endosomes with Ca^{2+} or calpain elicited AQP2 proteolysis, an effect abolished by leupeptin. This endogenous, Ca^{2+}-sensitive protease activity exhibited a different proteolytic digest pattern from trypsin, which also degraded AQP2 in vitro. IMCDs contain abundant μ-calpain protein and functional calpain proteolytic activity as demonstrated by immunohistochemistry, immunoblotting, and gel zymography. Furthermore, by small particle flow cytometry we demonstrated that μ-calpain colocalizes with apical IMCD endosomes. DHT does not appear to elicit general proteolysis, however, in addition to AQP2 degradation, DHT treatment also diminished μ-calpain and calpastatin expression although whether these changes contributed to the AQP2 instability remains unclear. Together, these data show for the first time that AQP2 is a substrate for calpain-mediated proteolysis and that furthermore, μ-calpain, like AQP2, is both highly expressed in renal inner medulla and localized to apical IMCD endosomes.

Keywords: Hypercalciuria; IMCD; Water channel; Protease

Vasopressin-elicited water reabsorption is mediated by the cAMP-dependent trafficking of AQP2 water channel-containing vesicles into the apical membrane of inner medullary collecting ducts (IMCDs) (reviewed in [1,2]). Polyuria resulting from downregulation of AQP2 has been described in several rat model experimental conditions including chronic lithium treatment [3], hypokalemia [4], low-protein diet [5], and chronic dihydrotachysterol (DHT) treatment [6,7]. In the case of DHT-induced hypercalcemia/hypercalciuria, we have shown previously that whilst there is an 87% reduction in inner medullary AQP2 protein content there is no significant change in the AQP2 mRNA content [6]. Thus, the loss of AQP2 content of cells after DHT treatment is apparently a post-nuclear phenomenon, however, the mechanism of this protein instability is unknown.

Calpain is an intracellular calcium-activated neutral protease with at least 2 isozymic forms: μ-calpain, activated at micromolar concentrations of calcium and m-calpain, activated at millimolar concentrations of calcium (reviewed in [8,9]). Calpain, which has a molecular weight of 80–110 kDa, is present in a variety of tissues including the brain, erythrocytes, and kidney [8]. Calpain-mediated proteolysis has been implicated in a variety of cell processes including parathyroid hormone degradation [10], thyrotropin-releasing hormone-induced selective downregulation of protein kinase C ε in pituitary cells [11], calcium-induced inositol (1,4,5)-triphosphate receptor degradation [12], prostaglandin $F_{2\alpha}$-induced oxytocin secretion from the corpus luteum [13],

* Corresponding author. Fax: 1-11-44-161-275-5600.
E-mail address: d.ward@man.ac.uk (D.T. Ward).

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and cell injury/death in the proximal tubules [14]. Accordingly, we investigated whether calpain is also capable of AQP2 degradation in vitro and furthermore whether it likely represents the physiological protease in IMCD responsible for AQP2 degradation in vivo.

The hormone $1,25(OH)_2D_3$ exerts significant effects on the whole body calcium metabolism including increased intestinal calcium absorption and renal calcium reabsorption. In addition, $1,25(OH)_2D_3$ also stimulates calpain activity in breast carcinoma cells [15] and increases μ-calpain protein expression in renal cell carcinoma [16]. Thus, we then examined whether DHT-induced AQP2 downregulation could be accounted for by changes in the expression of μ-calpain or its endogenous inhibitor calpastatin [17].

Materials and methods

Animals. All studies were performed on Male Sprague–Dawley rats (200–300 g; Charles River Laboratories, Cambridge, MA). For the hypercalcemia experiments, the rats were pair-fed as described previously [6] with either a control diet (Prolab RMH 3000) or a diet containing 4.25 mg/kg dihydrotachysterol (DHT) for 5 days and were given free access to water. The rats were sacrificed under sedation with intraperitoneal injection of sodium pentobarbital (5 mg/kg). Blood was obtained by cardiac puncture and analyzed for serum calcium. Urine was obtained by bladder puncture and analyzed for urine calcium and creatinine. The kidneys were collected and samples were prepared from the inner medulla as described below.

Antibodies and reagents. Anti-μ-calpain monoclonal antibody was obtained from Sigma. Anti-calpastatin polyclonal antibody was a generous gift from Takaomi Saido, Japan. The calpastatin blocking peptide was prepared by QCB Biochemicals. Tris was obtained from US biochemical; glycine was from J.T. Baker; SDS, acrylamide, ammonium persulfate, and 2-mercaptoethanol were from Bio-Rad. All other chemicals were from Sigma.

Immunoblotting. Rat kidney inner medullae from control and DHT-treated rats were homogenized in Hepes (12 mM, pH 7.6)/mannitol (300 mM) buffer containing multiple protease inhibitors and purified apical membrane endosomes were then isolated from the resulting homogenates, as described previously [18,19]. Cytosolic fractions (supernatant following 100,000*g* ultracentrifugation of post-mitochondrial supernatant) were also collected and all samples were stored at −80 °C, prior to solubilization in 5× Laemmli buffer. Immunoblotting was performed as described before [18] with equal protein loading demonstrated by staining with Ponceau S and where immunoreactivity was detected using ECL reagents and quantified by densitometry [18].

Immunohistochemistry. As previously described [6], rats were perfusion fixed, using freshly prepared 4% paraformaldehyde. Tissue samples were then embedded in OCT compound (Miles, Elkart), snap frozen in 2-methylbutane in liquid N_2, and stored at −70 °C until further use. Immunohistochemistry was then performed as previously described [6] using frozen sections (4 μm) cut from the tip of individual rat kidney inner medullae and stained with various antisera. Counterstaining was performed with methyl green (Fisher, Pittsburgh, PA).

Casein zymography. Calpain activity was quantified according to the method of Raser et al. [20]. Protein samples were resolved in non-denaturing polyacrylamide gels containing 0.2% (w/v) casein. The running buffer contained 1 mM EGTA to stabilize the calpains. After electrophoresis the gels were washed and incubated in an activation buffer containing 4 mM calcium and 10 mM DTT for 24 h. The gels were then stained with Coomassie blue to stain the intact casein, whereas the regions where casein degradation had occurred appeared clear.

Flow cytometry colocalization of the binding of anti-calpain antisera with F-dextran entrapped in rat renal papillary heavy endosomes. Analysis and colocalization of entrapped dextrans and antibody binding on an endosome-by-endosome basis were performed as described previously [19] using a Becton–Dickinson FACSVantage flow cytometer optimized for small particle resolution. Multiparameter data were collected as 2000 event list, mode files and analyzed using *CellQuest* (Becton–Dickinson, Mountain View, CA). Colocalization of the binding of anti-calpain antisera to endosomes containing entrapped fluorescein dextran (F-dextran) by flow cytometry was performed after loading endosomes with F-dextran by intravenous infusion of 50–75 mg of 10,000 kDa F-dextran 15 min prior to renal harvest as described previously [19].

Results

Purified IMCD endosomes contain a Ca^{2+}-sensitive AQP2 protease activity

To test whether AQP2 represents an in vitro substrate for calpain-mediated proteolysis, we measured the AQP2 protein content of IMCD endosomes incubated at 37 °C in the presence or absence of calcium-dependent calpain (Fig. 1A). We found that cotreatment of calpain and calcium caused substantial proteolysis of AQP2 in the vesicle preparation. However, even in the absence of exogenous calpain, we observed that addition of 5 mM calcium alone was sufficient to induce partial AQP2 degradation, indicating the presence of an endogenous metalloproteinase present in the vesicles. Next, we compared the AQP2 degradation caused by calcium/calpain with that produced by exogenous trypsin addition, since trypsin is also expressed in the kidney [21]. As it can be seen in Fig. 1B, the primary proteolytic product of AQP2 following trypsin digestion is a 20-kDa species that was not observed in the endosomes exposed only to calcium and/or calpain (Fig. 1A).

To investigate further the nature of calpain-mediated AQP2 proteolysis, we examined the concentration dependence of the calpain/calcium effect. Increasing the amount of calpain added from 1 to 10 U/reaction gave a substantial increase in the amount of AQP2 degradation seen after 1 h at 37 °C (Fig. 2A). In addition, we found that in the presence of 10 U calpain, increasing $CaCl_2$ concentration from 1 to 5 mM slightly increased the degree of AQP2 degradation (Fig. 2A).

Next we tested whether cotreatment with various protease inhibitors could attenuate calpain-mediated AQP2 hydrolysis (Fig. 2B). First we found that vesicles incubated on ice for 1 h (Fig. 2B, lane 2), even in the presence of calpain/calcium, contained more AQP2 protein than those incubated at 37 °C in the absence of added calpain/calcium, again demonstrating the presence of an endogenous protease activity. Adding either

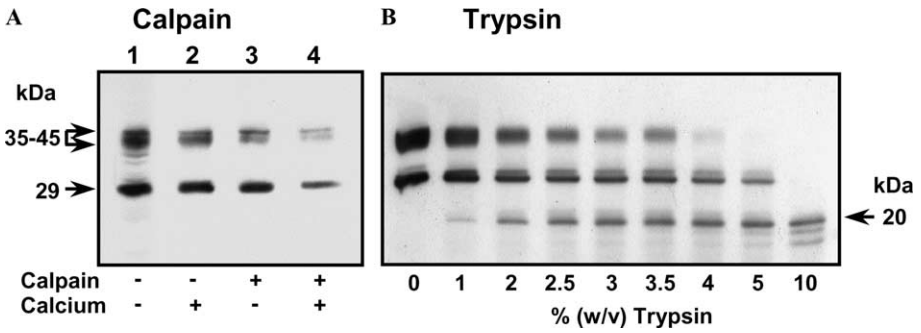

Fig. 1. Proteolysis of AQP2 by exogenous calpain and by an endogenous Ca^{2+}-activated protease. (A) Representative immunoblot showing decreased content of AQP2 in IMCD heavy endosomes incubated with purified calpain in the presence of calcium. IMCD heavy endosomes from the inner medulla were first washed to remove the protease inhibitors added during their preparation and four aliquots containing equivalent amounts of protein were then incubated for 1 h at 37 °C in the presence or absence of calcium (5 mM) and/or calpain (1 U). Note that the sample incubated with both calpain and calcium exhibits the maximum degradation (lane 4, $n = 8$) whilst the lanes containing either the calcium (lane 2) or calpain (lane 3) treatments alone show partial degradation. (B) Immunoblot showing the AQP2 content of IMCD endosomes incubated for 2.5 min at 37 °C in the presence of increasing amounts (1–10% w/v) of exogenous trypsin (Gibco-BRL). The tryptic digest of AQP2 produced primarily a 20-kDa product.

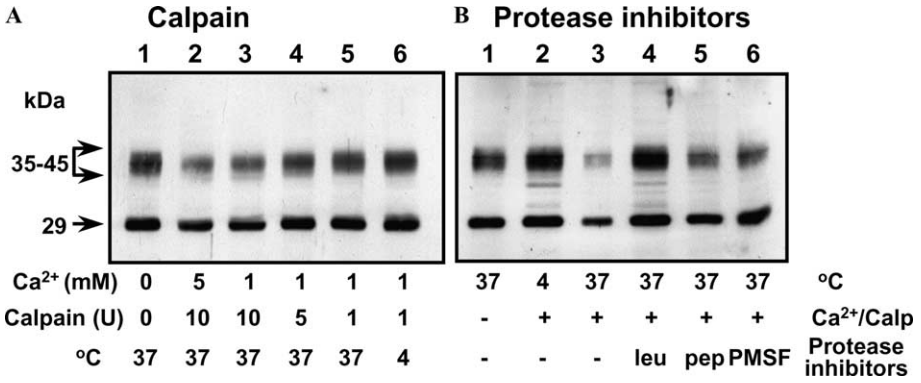

Fig. 2. The effect of exogenous calpain on AQP2 degradation is calcium- and concentration-dependent and strongly inhibited by leupeptin. Endosomes were incubated as described in the legend to Fig. 1. Panel A shows the AQP2 content of the vesicles following incubation at either 37 °C (lanes 1–5) or 4 °C (lane 6) in the presence or absence of various concentrations of Ca^{2+} and calpain. Endosomes where then incubated in buffer containing a fixed concentration of calpain (1 U/30 μl) and calcium (5 mM) but in the presence or absence of leupeptin (4 μM), pepstatin (1.25 μM), and PMSF (5 μM). Of the three protease inhibitors tested, leupeptin produced the greatest inhibition of calpain-mediated AQP2 proteolysis ($n = 3$).

pepstatin (lane 5) or PMSF (lane 6) both marginally inhibited the effect of the exogenous calpain, however, the addition of leupeptin completely ablated the AQP2 degradation, resulting both from the exogenous calpain activity and indeed the endogenous protease activity.

Rat IMCDs express abundant calpain protein including localization to apical endosomes

Next, we investigated the renal expression and localization of μ-calpain protein using a specific polyclonal antibody. Fig. 3A is an immunoblot showing that the highest expression of μ-calpain in the kidney is in the inner medulla. Representative light microscopy immunohistochemical stains confirm that rat inner medulla contains calpain (Fig. 3B) and also the endogenous μ-calpain inhibitor calpastatin (Fig. 3C). The antibody staining for both proteins is highest in the collecting ducts and includes immunoreactivity in the region of the apical membrane.

The distribution and binding of anti-calpain antisera to rat renal papillary heavy endosomes were next quantified by flow cytometry using endosomes prepared from rats injected with fluorescein dextran (F-dextran) [19]. As shown in panel A of Fig. 4, purified endosomes exhibit a low level of auto-fluorescence and minimal binding occurred when using non-optimal antisera dilutions (not shown). In contrast, endosomes containing entrapped F-dextran were highly fluorescent at pH 8 (Fig. 4, panel B), although not at pH 4 (panel C). Since F-dextran fluorescence is pH sensitive this confirms that the source of the fluorescence is the entrapped fluorophore. Using the optimal anti-calpain antibody dilution (panel D), we observed that the endosomes were almost entirely positive for both F-dextran fluorescence and anti-μ-calpain binding (panel D), demonstrating that the

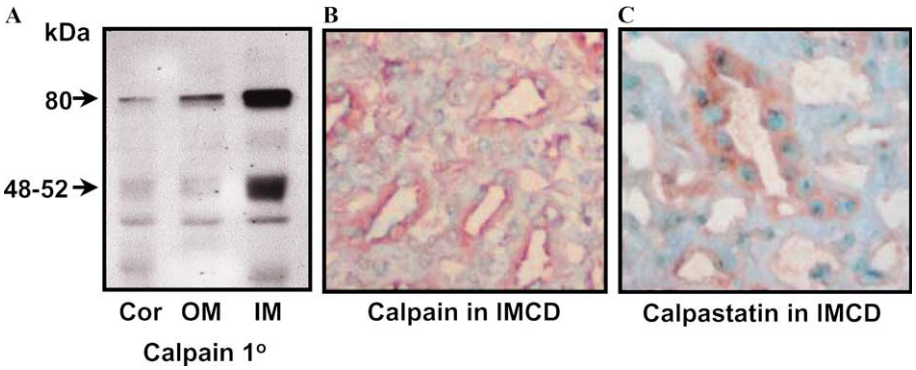

Fig. 3. Immunolocalization of μ-calpain and calpastatin to the IMCDs. (A) Western blot showing total homogenate (post-nuclear supernatant) from the cortex, outer medulla, and inner medulla of rat kidneys (25 μg protein/lane) immunoblotted against anti-μ-calpain monoclonal antibody (1:5000). Calpain content as depicted by the 80-kDa band was found to be highest in the inner medulla. (B,C) Representative light microscopy immunohistochemical stains showing that IMCDs contain μ-calpain (B) and calpastatin (C). Specific antibody binding is indicated by the rose-colored reaction product (magnification, 2500×).

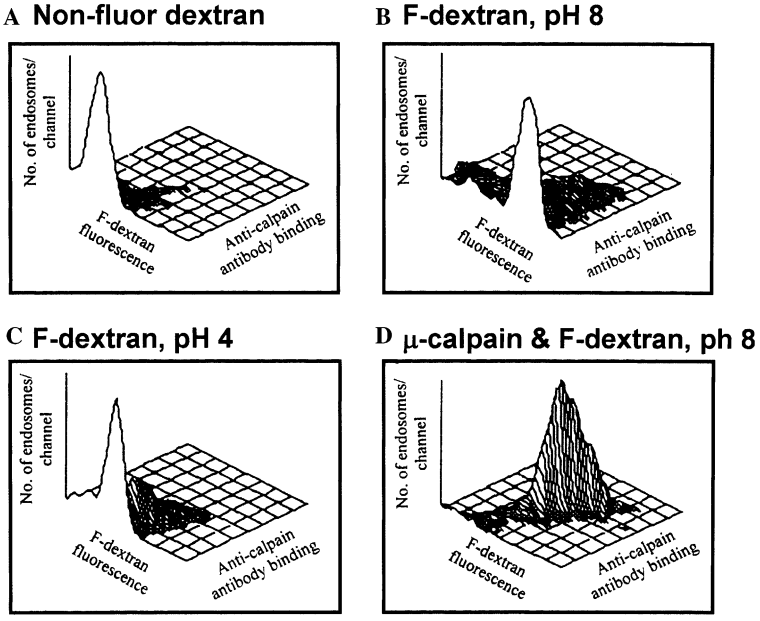

Fig. 4. Colocalization of anti-calpain binding with F-dextran fluorescence in a population of individual endosomes. Using flow cytometry, the magnitudes of fluorescein fluorescence (*X*-axis) and binding of affinity-purified antisera detected by phycoerythrein-conjugated secondary antisera (*Y*-axis) were quantified for 2000 AQP2 endosomes. Number of endosomes per channel is displayed up out of the page (Z-axis). Note that both *X* and *Y* axes are log scales. (A) Rat renal papillary heavy endosomes prepared with non-fluorescent dextran and labelled with non-optimal antisera dilutions demonstrate autofluorescence and non-specific binding. (B) Entrapped F-dextran equilibrated to optimum pH of 8.0 shows more than a log shift in fluorescein fluorescence. (C) The F-dextran can be quenched by incubation at pH 4.0 showing a dynamic signature consistent with entrapped dextran. (D) F-dextran and anti-calpain antibody binding at optimal dilution demonstrates colocalization of the two markers and near uniform membrane binding.

μ-calpain localizes to an apically derived fraction of IMCD endosomes.

DHT-induced changes in IMCD protein expression

Previously we have shown that IMCD apical endosomes purified from DHT-treated rats exhibit significantly reduced AQP2 content. To test whether this observed reduction in AQP2 is merely a general reduction in protein content of AQP2 containing endosomes or relatively specific to AQP2, we prepared apical IMCD endosomes from DHT-treated rats and quantified their protein content of a number of proteins. First, we confirmed that chronic DHT treatment had induced hypercalcemia in the animals (total serum calcium concentration; control, 10.0 ± 0.1 mg/dl; DHT 13.3 ± 0.2 mg/dl; $N = 7$, $P < 0.001$ by Student's t test; Tufts Veterinary Diagnostic Laboratory, Grafton, MA)

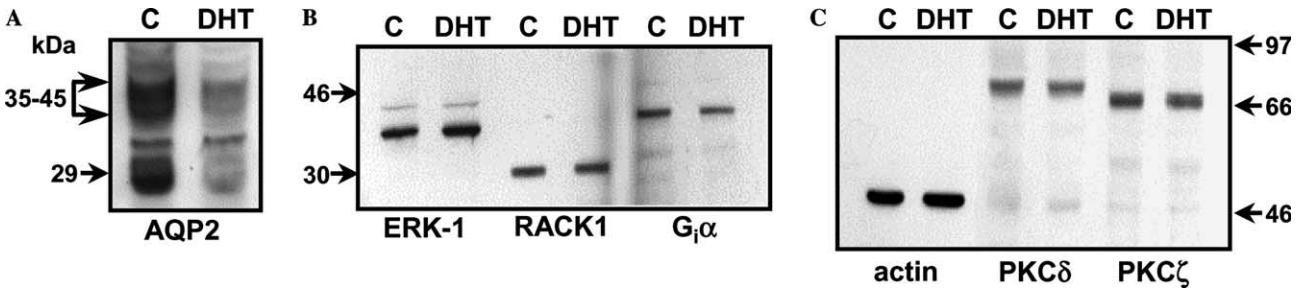

Fig. 5. DHT-induced AQP2 downregulation is not a generalized effect on all IMCD proteins. Endosomes obtained from cell lysates from the inner medulla of control and DHT-treated rats were pair-immunoblotted with antibodies to AQP2, ERK1, RACK1, $G\alpha_i$, actin, PKCδ, and PKCζ. Substantial DHT-induced downregulation was limited, in this panel of proteins, to AQP2.

consistent with our previous experiments [6]. Next, the endosomes were pair-immunoblotted using antibodies to AQP2, extracellular signal-regulated kinase (ERK)1, the protein kinase C (PKC)-anchoring protein RACK1, the G protein $G\alpha_i$, actin, and the PKC isoforms δ and ζ (Fig. 5). The greatest reduction in the endosomal content of any of these proteins was in AQP2, confirming our previous data. There was a small reduction in the presence of $G\alpha_i$, a marginal reduction in the presence of PKCδ and PKCζ, and no change at all in the levels of ERK1, RACK1, and actin.

We finally examined whether the effect of DHT-induced AQP2 downregulation can be accounted for by altered expression of either μ-calpain or its endogenous inhibitor calpastatin. After 5 days of DHT treatment, we observed decreased protein expression of μ-calpain by immunoblotting (Fig. 6A), reduced calpain activity by zymography (Fig. 6B), and decreased expression of calpastatin (Fig. 6).

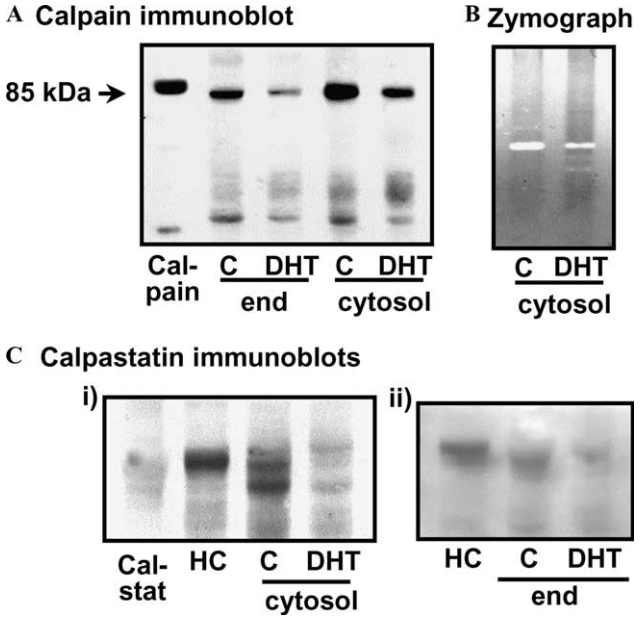

Fig. 6. Effect of DHT-treatment on IMCD expression of calpain and calpastatin. (A) Endosomal and cytosolic fractions were prepared from inner medullary lysate from control (C) and DHT-treated (D) rats and 25 μg protein loaded in each well. The resulting μ-calpain immunoblot reveals an apparent decrease in μ-calpain expression (80 kDa band) following DHT treatment in both the cytosolic and endosomal fractions. (B) Casein zymography was performed as described in Materials and methods using 100 μg protein from the cytosolic fractions of the cell lysates. The calpain band shown in this non-denatured PAGE gel comigrated with exogenous calpain protein (not shown). The samples derived from the DHT-treated rats exhibited reduced calpain activity. (C) Using different aliquots from the same samples shown in Panel A, samples were immunoblotted using anti-calpastatin polyclonal antibody (1:800). Control cytosolic fractions contained 68% more calpastatin than DHT treatment-derived samples. An apparent reduction in calpastatin content was also observed in endosomes.

Discussion

In the current study, we demonstrate that rat IMCDs and in particular apically derived, AQP2-containing heavy endosomes [19] contain abundant μ-calpain protein and a calcium-activated proteolytic activity that is capable of degrading AQP2. We first demonstrated that the AQP2 in IMCD endosomes is a substrate both for exogenous calpain and an endogenous metalloproteinase activity present within the vesicles. The pattern of proteolytic digestion resulting from exposure to calcium ions was similar to that resulting from the addition of calpain (Fig. 1A). In contrast, degradation of endosomal AQP2 with trypsin, an enzyme that is highly expressed in renal collecting ducts [21], produced a 20-kDa AQP2-immunoreactive species not seen with the endogenous protease (Fig. 1B). Therefore, although these data demonstrate that trypsin is an AQP2 protease at least in vitro, the absence of the 20-kDa AQP2 tryptic product from purified endosomes incubated at 37 °C (Fig. 1A) would tend to suggest that it is not responsible for the AQP2 proteolysis we observed in vivo.

The calpain-mediated AQP2 degradation was completely ablated by cotreatment of IMCD homogenates with leupeptin (Fig. 2), supporting the findings of Sorimachi et al. [22] who showed that leupeptin is a potent inhibitor of calpain activity. Indeed, leupeptin addition abolished not only the AQP2 degradation caused by the

exogenous calpain but prevented all AQP2 proteolysis including that caused by the endogenous protease activity. Whilst we cannot rule out the possibility that in addition to calpain, further AQP2 protease activities may exist within the endosomes, Fig. 2B shows that such enzymes must be leupeptin-sensitive.

We found that renal μ-calpain expression is highest in inner medulla and within the inner medulla is highest in the collecting ducts (Fig. 3). The μ-calpain immunoreactivity was present throughout the IMCD cells including localization at the apical membrane. Furthermore, colocalization studies using small particle flow cytometry revealed that μ-calpain immunoreactivity was associated with F-dextran-containing IMCD heavy endosomes (Fig. 4). These IMCD heavy endosomes have been extensively characterized and shown previously to be of apical origin, to possess abundant AQP2 protein, and to exhibit substantial, mercurial-sensitive osmotic water permeability [19,23]. Thus, the current data suggest that the same vesicles also contain (1) μ-calpain protein, (2) endogenous calpain activity based on the casein protease in-gel zymography, and (3) a calcium-activated proteinase activity capable of degrading AQP2. Since we also demonstrated that AQP2 is a substrate for exogenous calpain then, together, these data suggest that calpain could represent an in vivo proteolytic modulator of AQP2 content.

Since AQP2 protein becomes downregulated in hypercalcemic rats without an alteration in AQP2 mRNA expression [6], we next examined whether calpain is the protease specifically responsible for the AQP2 downregulation following DHT treatment. For example, upregulation of μ-calpain protein or downregulation of its endogenous inhibitor, calpastatin, are two molecular mechanisms that could theoretically contribute to DHT-induced AQP2 downregulation. What we observed, however, was more complicated than this. That is, although DHT did cause an apparent decrease in calpastatin expression, there was also an apparent reduction in μ-calpain expression and calpain activity in IMCD. Nevertheless, the magnitude of these changes is interesting given the fact that AQP2 is also substantially downregulated following DHT treatment whereas we saw little change in the expression of six other IMCD vesicle proteins (Fig. 5).

If calpastatin downregulation precedes μ-calpain downregulation then, for a time, calpain activity in vivo may become elevated and contribute to AQP2 degradation. However, if calpain downregulation precedes AQP2 degradation, then the reverse would be true. As the time course for each molecular change was not determined in the current study no firm conclusion can be drawn regarding the significance of calpain/calpastatin downregulation to AQP2 downregulation. In any case, calpain activity could be elevated in vivo without necessarily observing the upregulation of calpain or downregulation of calpastatin. As a calcium-dependent protease, sustained or repeated elevation of intracellular Ca^{2+} levels arising from the chronic hypercalcemia/hypercalciuria may be sufficient to stimulate and/or maintain calpain activation. Although this remains to be positively demonstrated, the μ-calpain/calpastatin downregulation reported here may be at least suggestive of chronic activation of this proteolytic pathway.

In summary, the current data show for the first time that AQP2 is a substrate for calpain and that μ-calpain is both highly expressed in IMCD and localized to the same endosomal fraction that contains AQP2. Furthermore, these IMCD endosomes exhibit an endogenous calpain-like activity that results in AQP2 proteolysis. Thus, if calpain is an AQP2 protease in vivo, it will be interesting to discover whether it represents a housekeeping protease maintaining normal, physiological levels of the water channel, or, if its primary role is to modulate AQP2 levels when intracellular calcium levels are elevated.

Acknowledgments

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