Methyl-β-cyclodextrin induces vasopressin-independent apical accumulation of aquaporin-2 in the isolated, perfused rat kidney

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Russo, Leileata M., Mary McKee, and Dennis Brown. Methyl-β-cyclodextrin induces vasopressin-independent apical accumulation of aquaporin-2 in the isolated, perfused rat kidney. Am J Physiol Renal Physiol 291: F246–F253, 2006. First published January 31, 2006; doi:10.1152/ajprenal.00437.2005.—Vasopressin increases urine concentration by stimulating plasma membrane accumulation of aquaporin-2 (AQP2) in collecting duct principal cells, allowing bulk water flow across the collecting duct from lumen to interstitium down an osmotic gradient. Mutations in the vasopressin type 2 receptor (V2R) cause hereditary X-linked nephrogenic diabetes insipidus (NDI), a disease characterized by excessive urination and dehydration. Recently, we showed that inhibition of endocytosis by the cholesterol-depleting drug methyl-β-cyclodextrin (mβCD) induces plasma membrane accumulation of AQP2 in transplanted renal epithelial cells overexpressing epitope-tagged AQP2. Here, we ask whether mβCD could induce membrane accumulation of AQP2 in situ using the isolated, perfused kidney (IPK). By immunofluorescence and electron microscopy, we show that AQP2 was shifted from a predominantly intracellular localization to the apical membrane of principal cells following 1-h perfusion of Sprague-Dawley rat kidneys with 5 mM mβCD. Quantification of staining revealed that the intensity of AQP2 was increased from 647 ± 114 (control) to 1,968 ± 299 units (mβCD; P < 0.001), an effect similar to that seen after perfusion with 4 nM dDAVP (1,860 ± 298, P < 0.001). Similar changes were observed following mβCD perfusion of kidneys from vasopressin-deficient Brattleboro rats. No effect of mβCD treatment on the basolateral distribution of AQP3 and AQP4 was detected. These data indicate that AQP2 constitutively recycles between the apical membrane and intracellular vesicles in principal cells in situ and that inducing apical AQP2 accumulation by inhibiting AQP2 endocytosis is a feasible goal for bypassing the defective V2R signaling pathway in X-linked NDI.

diabetes insipidus; collecting duct; endocytosis; immunocytochemistry; aquaporin recycling

AQUAPORIN-2 (AQP2) is a hydrophobic ~29-kDa protein that is composed of six transmembrane segments and cytoplasmically located NH2 and COOH termini (2). Expressed in principal cells of the kidney collecting duct (CD), AQP2 plays a major role in whole body water homeostasis through water reabsorption that results in urine concentration (6, 8). This function is achieved by the insertion of AQP2 into the apical membrane of principal cells, where it acts as a hydrophilic pore allowing the osmotic flow of water into the cell. Water then exits basolaterally into the interstitium via aquaporins-3 and/or -4 (12, 35) and perhaps also AQP2 in some kidney regions (9, 17a, 25).

Dysfunction of the AQP2 membrane accumulation process results in whole body deregulation of water homeostasis, contributing to excessive urination and dehydration, a disease known as diabetes insipidus (DI) (10, 24, 26, 31). AQP2 distribution and function are chiefly regulated by the antidiuretic hormone arginine vasopressin (AVP), which is released by the posterior pituitary gland in response to high-serum osmolarity and/or blood volume reduction (17). Circulating AVP binds to the vasopressin type 2 receptor (V2R), located on the basolateral side of principal cells of the CD, causing intracellular cAMP levels to increase. This activates protein kinase A (PKA) (27), resulting in the phosphorylation of AQP2 at residue S256. This phosphorylation is necessary for the AVP-induced redistribution of AQP2 from a vesicular to a membrane localization (13, 18), allowing water reabsorption to occur in the CD. AQP2 membrane accumulation can also occur after an increase in cytoplasmic cGMP levels; protein kinase G (PKG) is also capable of phosphorylating AQP2 on residue S256 (4).

DI has several distinct causes. Hereditary forms may be “nephrogenic (NDI)” (the result of mutations in the AQP2 or V2R genes) or “central (CDI)” (the result of mutations in the AVP gene). Several mutations in the AQP2 gene have been identified and they commonly lead to the mistargeting of AQP2 to subcellular organelles rather than the plasma membrane, resulting in AQP2 degradation (23). X-linked NDI is caused by mutations in the V2R and is much more common than autosomal NDI in which AQP2 is defective. The AQP2 protein expressed in X-linked NDI patients as well as in patients with CDI is probably functional, but the inability of the kidney to respond to AVP or the lack of active AVP hormone precludes AQP2 accumulation on the plasma membrane of principal cells.

In cases where AVP itself is dysfunctional, the symptoms of CDI may be treated by administration of 1-desamino(8-D-arginine)vasopressin or dDAVP, a synthetic structural analog of AVP that has a high specificity for the V2R (1). Treatment of CDI with dDAVP is generally successful; however, dDAVP in combination with some drugs may cause dilutional hyponatraemia (12a). Of course, dDAVP is not effective in most cases where the V2R receptor is mutated or unresponsive to AVP, although high doses of AVP may be effective in a few instances where the V2R has a lower, but not absent, affinity for the hormone. To date, therefore, the treatment of NDI resulting from V2R mutations is limited and relies largely on reducing urine flow by (seemingly paradoxical) treatment with thiazide diuretics with concomitant sodium restriction.

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However, some recent studies are emerging that suggest alternative strategies for therapeutic intervention in NDI. These are based on our increasing understanding of the signal transduction and trafficking pathways that result in the membrane accumulation of AQP2. Previous work in vitro showed that AQP2 is constitutively recycled between the plasma membrane and intracellular vesicles, even in the absence of AVP (22, 32). The relative rates of endo- and exocytosis of AQP2 were proposed to be critical in determining the amount of AQP2 in the plasma membrane (14, 25). After reaching the cell surface, AQP2 is subsequently internalized by clathrin-mediated endocytosis (32). The depletion of plasma membrane cholesterol by methyl-β-cyclodextrin (mβCD) prevents the formation and budding of clathrin-coated pits, resulting in the acute blockade of endocytosis (29). With the use of this strategy, a very rapid plasma membrane accumulation of AQP2 was demonstrated in AQP2-transfected LLC-PK1 and inner medullary CD (IMCD) cells in culture. Furthermore, inhibiting endocytosis with dominant-negative (K44A) dynamin also resulted in membrane accumulation of AQP2 in cultured cells (32).

The aim of our present study was to determine whether the endocytosis inhibitor mβCD would cause vasopressin-independent AQP2 accumulation in principal cells of the functioning, intact rat kidney. This would indicate that AQP2 is constitutively recycling not only in transfected cell models in vitro but also in CD principal cells in situ. However, mβCD...
targets and binds cholesterol, and its use in vivo is limited due to its potential toxicity and its ability to lyse erythrocytes. Therefore, we examined the effect of mβCD on AQP2 localization using the ex vivo isolated, perfused kidney technique (IPK). This technique allows the kidney to function on an artificial closed circuit, hence removing extra renal influences, making the IPK an invaluable tool for the investigation of drugs or compounds that may be toxic or display complex interactions in vivo (33). The results obtained confirm previous in vitro studies and demonstrate that AQP2 constitutively recycles to the apical membrane, a process that is halted by the depletion of cholesterol using mβCD and which results in the accumulation of AQP2 on the plasma membrane (22). This provides proof-of-principle evidence that specific targeting of mβCD (or other agents that inhibit endocytosis) to CDs may provide a novel form of treatment for DI resulting from dysfunctional V2R or AVP or as an alternative to dDAVP treatment in cases where its use is contraindicated.

MATERIALS AND METHODS

IPK preparation. All animal experiments carried out were approved by the Institutional Committee on Research Animal Care, in accordance with National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats or male Brattleboro rats weighing 250–270 g were used. To prevent the interference of oxytocin release on AQP2 distribution during surgery (19), Brattleboro rats (which lack vasopressin) were deprived of water overnight before the experiment. Rats were anesthetized by an intraperitoneal injection of 45 mg/kg body wt of pentobarbital sodium (Nembutal; Abbott Laboratories, Chicago, IL). PBS containing 10% mannitol and 200 IU heparin (final volume 1 ml; Sigma, St. Louis, MO) was injected into the femoral vein. To prevent dehydration, 1.5 ml of 0.9% NaCl were also injected into the femoral vein immediately before surgery. A laparotomy was performed and the right ureter was catheterized with polyethylene tubing PE/1 (Scientific Commodities). The right renal artery was cannulated via the superior mesenteric artery and the kidney was removed by en bloc dissection. The whole procedure was completed within 10 min. The kidney perfusion pressure was maintained at 95–100 mmHg with a peristaltic pump monitored using a calibrated aneroid manometer. The kidney was perfused with a recirculating Krebs-Henseleit buffer containing 5% BSA,
glucose, essential amino acids, and oxygen radical scavengers as previously described (34). The perfusate was filtered using a 0.45-μm filter and was oxygenated with 95% O₂-5% CO₂, pH 7.4, and maintained at 37°C throughout the entire procedure. The kidney was first equilibrated on the apparatus for 10 min and was then perfused for a 60-min time period. Urine and plasma samples were collected at 40 and 60 min. Urine osmolality was measured using a Wescor Vapor Pressure Osmometer 5520 (Wescor, Logan, UT).

mβCD and dDAVP experiments were carried out using exactly the same procedure except that the perfusate contained 5 mM mβCD (Sigma) or 4 nM dDAVP (Sigma).

**Tissue fixation and immunostaining.** Following perfusion on the IPK apparatus, the kidney was immediately flushed with paraformaldehyde lysine periodate (PLP) fixative and then immersion-fixed overnight in PLP, as described previously (3). Tissue was then extensively washed in PBS and cryoprotected in 30% sucrose. Tissue was embedded in OCT and 5-μm-thick cryosections were cut. Sections were treated with 1% SDS in PBS, an antigen retrieval technique (7), and then blocked using a solution of 1% BSA in PBS. Previously characterized primary antibodies used were against the COOH terminus of AQP2 (30), AQP3, or AQP4 (15). Secondary antibodies used were goat anti-rabbit IgG coupled to CY3 or FITC (Jackson ImmunoResearch Laboratories, West Grove, PA).

Staining was analyzed using a Nikon E800 fluorescence microscope equipped with a Hamamatsu Orca CCD camera and IP Lab Spectrum acquisition and image analysis software, or with a Zeiss Axioplan microscope equipped with a Radiance 2000 confocal laser-scanning system (Bio-Rad). For quantification, digital images were acquired using the Nikon microscope with a ×40 objective. All images from different slides were collected with the same microscope setting. Perpendicularly sectioned cells from the inner stripe/inner medulla boundary region were chosen at random and a line was drawn through the cell from the basolateral to apical pole, passing through the region of the nucleus. The line was analyzed for intensity and the intensity at the level of the apical pole was recorded. Twenty-five individual cells were quantified in each of three to four kidneys from each group (75–100 points in total for each group). Analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. NIH and available on the Internet at http://rsb.info.nih.gov/nih-image/).

**Electron microscopy and immunogold staining.** For electron microscopy and immunogold staining, small pieces of PLP-perfused rat kidney (<1 mm³) were dehydrated through a graded series of ethanol to 100% ethanol. They were then infiltrated with LR White resin (Electron Microscopy Sciences, Fort Washington, PA) and were placed in fresh LR White embedding resin for electron microscopy analysis using immunogold labeling for AQP2. AQP2 expression was greatly increased in the mβCD-treated kidney (Fig. 1C), an effect similar to that of dDAVP (Fig. 1E). This marked apical redistribution of AQP2 induced by both mβCD and dDAVP was also evident in CDs of the cortex and the outer stripe (Figs. 2 and 3). In the middle portion of the papilla, AQP2 was also relocated to the apical pole of principal cells following mβCD treatment, whereas dDAVP induced not only apical accumulation, but also basolateral localization of AQP2 in this portion of the CD (Fig. 1, B, D, F), as previously described in vivo (36). In addition, basolateral AQP2 was also seen in a population of cortical connecting segments (data not shown), a phenomenon that has also been described earlier (9). The AQP2 redistribution was also evident by electron microscopy (Fig. 4, A–C), where AQP2 gold labeling showed an increased apical plasma membrane localization following mβCD (Fig. 4B) and dDAVP (Fig. 4C) treatment. However, a marked basolateral accumulation of AQP2 was noted after dDAVP, but not mβCD treatment in the middle portion of

**RESULTS AND DISCUSSION**

Following perfusion of intact, isolated kidneys with 5 mM mβCD for 60 min, immunocytochemical analysis of AQP2 revealed a striking redistribution of AQP2. It relocated from a predominantly cytoplasmic location in outer medullary (inner stripe) principal cells of the nontreated IPK (Fig. 1A) to an apical distribution in the mβCD-treated kidney (Fig. 1C), an effect similar to that of dDAVP (Fig. 1E). This marked apical redistribution of AQP2 induced by both mβCD and dDAVP was also evident in CDs of the cortex and the outer stripe (Figs. 2 and 3). In the middle portion of the papilla, AQP2 was also relocated to the apical pole of principal cells following mβCD treatment, whereas dDAVP induced not only apical accumulation, but also basolateral localization of AQP2 in this portion of the CD (Fig. 1, B, D, F), as previously described in vivo (36). In addition, basolateral AQP2 was also seen in a population of cortical connecting segments (data not shown), a phenomenon that has also been described earlier (9). The AQP2 redistribution was also evident by electron microscopy (Fig. 4, A–C), where AQP2 gold labeling showed an increased apical plasma membrane localization following mβCD (Fig. 4B) and dDAVP (Fig. 4C) treatment. However, a marked basolateral accumulation of AQP2 was noted after dDAVP, but not mβCD treatment in the middle portion of...
the inner medulla (not shown). Morphologically, the CDs from all IPK groups looked similarly intact, and epithelial cells were well preserved when analyzed by electron microscopy, demonstrating that the change in AQP2 distribution using mβCD treatment was not accompanied by gross cellular alterations. In support of this, both AQP3 and AQP4 maintained their usual tight basolateral localization in both the nontreated control and mβCD-treated IPK (Fig. 5, A-D).

Analysis of AQP2 distribution in the apical pole of principal cells from CDs along the inner stripe/inner medullary border (where the effect was the most pronounced) using NIH Image revealed the apical AQP2 redistribution to be statistically significant in both mβCD-treated group (P < 0.001) and dDAVP-treated group (P < 0.001) compared with the nontreated control IPK (Fig. 6). Both treatments resulted in an almost threefold increase in apical membrane staining intensity in this system. However, despite this increase in apical AQP2, no significant change in urine concentrating ability was observed [330 ± 11 (n = 4) control IPK vs. 362 ± 6.8 (n = 3) dDAVP-treated IPK]. This is consistent with the results of Lieberthal et al. (21), who demonstrated that the addition of dDAVP resulted in the partial production of concentrated urine by the IPK only when erythrocytes (40–45% hematocrit) were included in the circulating medium (20). This maneuver was not possible in the present set of experiments, because mβCD efficiently lyses erythrocytes, which can lead to tubular necrosis due to the release of heme proteins (5).

To determine whether the same effects of mβCD could be obtained in a vasopressin-deficient model in which AQP2 levels are considerably lower than in normal rats (28), Brattleboro rats which lack functional AVP were used in a parallel study to determine the effect of mβCD on AQP2 distribution. Our data revealed a similar redistribution of AQP2 from a cytoplasmic localization to an apical localization following 5 mM mβCD perfusion in these animals (Fig. 7, A and B). Similar to the results from Sprague-Dawley rats, AQP3 distri-
bution (Fig. 7, C and D) and AQP4 distribution (not shown) were also unaffected by the perfusion with mβCD in the Brattleboro rat kidney. This finding demonstrates that even in the absence of preexposure to AVP in vivo, AQP2 can be sequestered to the apical membrane of principal cells by exposure to mβCD.

The Sprague-Dawley-derived IPK perfused with 5 mM mβCD displayed a decrease in kidney flow rate and urine production (Table 1), although perfusion pressure was not significantly different from nontreated control IPKs (Table 1). Urine osmolality showed a tendency to be higher in the mβCD-treated rat kidneys (as also seen for the dDAVP-treated rats), although this was not statistically significant (Table 1). As mentioned above, this finding is in agreement with previous data suggesting that the IPK has a compromised concentrating ability (20, 21). Although attempts were also made to analyze the effect of mβCD on endocytosis through the injection of a concentrated bolus of FITC dextran or horseradish peroxidase (HRP) into the renal artery, we were unable to visualize the endocytosis of these markers in any of the three groups studied.

Previous reports indicate that the IPK has disrupted sodium handling as well as progressive ischemic injury of the medullary thick ascending limb contributing to a decreased concentrating ability (20, 21). In the present study, this decreased concentrating ability also hampered efforts to analyze endocytosis of the fluid phase markers FITC-dextran and HRP as they did not achieve a high enough concentration in the renal tubule to permit ready visualization.

Our data demonstrate for the first time the ability of mβCD to sequester AQP2 in the apical membrane of CD principal cells in the intact ex vivo functioning kidney. The addition of mβCD to the IPK perfusate was able to significantly increase the level of apical AQP2 in the CD in all kidney regions, without affecting the expression and localization of AQP3 or AQP4. However, the effect was most pronounced in the inner stripe and the initial two-thirds of the inner medulla. A similar effect was also found in kidneys from the vasopressin-deficient Brattleboro rat. These data are consistent with the idea that

**Table 1. Physiological parameters for IPK perfusion of Sprague-Dawley rat kidneys**

<table>
<thead>
<tr>
<th>Time</th>
<th>Pressure, mmHg</th>
<th>Flow Rate, ml/min</th>
<th>Urine Volume, ml</th>
<th>Plasma Osmolarity, mosM</th>
<th>Urine Osmolarity, mosM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>M</td>
<td>C</td>
<td>M</td>
<td>C</td>
</tr>
<tr>
<td>40 min</td>
<td>94±2.5</td>
<td>99±0.5</td>
<td>21.8±2.3</td>
<td>7.0±1.5*</td>
<td>0.92±0.3</td>
</tr>
<tr>
<td>20 min</td>
<td>96±2.5</td>
<td>100±1</td>
<td>20.7±2.4</td>
<td>6.7±1.6*</td>
<td>0.65±0.2</td>
</tr>
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</table>

Values are means ± SE, n = 4 for each group. Where time is perfusion time, C is control isolated, perfused kidney (IPK) and M is methyl-β-cyclodextrin IPK. *P < 0.001, †P < 0.05 compared with control IPK.
AQP2 constitutively recycles between cytoplasmic vesicles and the apical plasma membrane of principal cells of the kidney, in the absence of vasopressin, a process that is regulated at least in part by clathrin-mediated endocytosis (22, 32). However, the observation that dDAVP but not mβCD can also induce basolateral accumulation of AQP2 in some regions of the CD implies that while they are similar, the mechanisms of action of the hormone and the drug may not completely overlap.

Although we previously reported that AQP2 constitutively recycles in LLC-PK1 and IMCD cells in culture (22, 32), the fact that this constitutive recycling process occurs in situ is an important finding for the development of future therapies for NDI. The ability of AQP2 to be sequestered in the apical membrane is paramount for the CD to exert its water reabsorption function in the kidney. Although mβCD is toxic in vivo, our present findings provide solid support for the idea that specifically blocking apical endocytosis in the CD of nephrogenic DI patients may eventually provide a plausible form of therapy for NDI.

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