Inhibition of endocytosis causes phosphorylation (S256)-independent plasma membrane accumulation of AQP2

Hua Lu,* Tian-Xiao Sun,* Richard Bouley, Karen Blackburn, Margaret McLaughlin, and Dennis Brown. Inhibition of endocytosis causes phosphorylation (S256)-independent plasma membrane accumulation of AQP2. *Am J Physiol Renal Physiol* 286: F233–F243, 2004. First published September 30, 2003; 10.1152/ajprenal.00179.2003.—Inhibition of clathrin-mediated endocytosis by expression of a GTPase-deficient dynamin mutant (dynamin-2/K44A) for 16 h results in an accumulation of plasma membrane aquaporin-2 (AQP2) in epithelial cells stably transfected with wild-type AQP2. We now show a similar effect of K44A dynamin in LLC-PK1 cells transfected with an S256 phosphorylation-deficient AQP2 mutant, AQP2(S256A), and in AQP2-transfected inner medullary collecting duct (IMCD) cells. More acute blockade of endocytosis in these cells with the cholesterol-depleting agent methyl-β-cyclodextrin (mβCD; 10 mM) resulted in a rapid and extensive cell-surface accumulation of both wild-type AQP2 and AQP2(S256A) within 15 min after treatment. This effect was similar to that induced by treatment of the cells with vasopressin. Blockade of endocytosis by mβCD was confirmed using quantitative analysis of FITC-dextran uptake and AQP2 membrane insertion was verified by cell-surface biotinylation. These data indicate that AQP2 recycles constitutively and rapidly between intracellular stores and the cell surface in LLC-PK1 and IMCD cells. The constitutive trafficking process is not dependent on phosphorylation of the serine-256 residue of AQP2, which is, however, an essential step for regulated vasopressin/cAMP-mediated translocation of AQP2. Our data show that rapid and extensive plasma membrane accumulation of AQP2 can occur in a vasopressin receptor (V2R)- and phosphorylation-independent manner, pointing to a potential means of bypassing the mutated V2R in X-linked nephrogenic diabetes insipidus to achieve cell surface expression of AQP2.

inner medullary collecting duct; aquaporin-2; methyl-β-cyclodextrin

THE ANTI DIURETIC hormone vasopressin (VP) stimulates urinary concentration by increasing the transepithelial water permeability of kidney collecting ducts. A considerable amount of work over the past two decades has established that VP functions by recruiting the aquaporin-2 (AQP2) water channel from cytoplasmic vesicles to the plasma membrane of collecting duct principal cells, but several aspects of this process are still not well understood. The classical hypothesis is that AQP2 recycles between cytoplasmic vesicles and the cell surface in a regulated pathway that is modulated by cAMP elevation in response to VP stimulation. Activation by cAMP of protein kinase A (PKA) leads to phosphorylation of PKA substrates, including AQP2. Phosphorylated AQP2 then translocates from intracellular vesicles to the apical plasma membrane of principal cells (3, 4, 11, 26, 31).

Increasing evidence, however, shows that cell-surface accumulation of AQP2 can be induced by other maneuvers that appear to be independent of cAMP. One such pathway in outer medullary principal cells and in cultured cells is stimulated by cGMP elevation in response to sodium nitroprusside, atrial natriuretic peptide, and t-arginine (2). The common feature of both cAMP- and cGMP-mediated AQP2 trafficking is that it requires protein phosphorylation at residue S256 in the AQP2 COOH terminus (2, 12, 20). Other studies have indicated that the actin cytoskeleton is also involved in regulating AQP2 recycling (14) and that actin depolymerization alone can lead to the cell-surface accumulation of AQP2 in the absence of any hormonal stimulus (22). Furthermore, a critical role of calcium in AQP2 translocation has been demonstrated (8).

In addition, studies on AQP2 trafficking in cultured epithelial cells have shown that AQP2 moves continually between the cell surface and intracellular vesicles in a constitutive recycling pathway that is independent of hormonal stimulation (13). During the endocytotic portion of its recycling pathway, AQP2 accumulates in clathrin-coated pits and is internalized via a clathrin-mediated process (5, 6, 37, 39). Our recent work showed directly that AQP2 accumulates on the plasma membrane in a VP-independent manner when clathrin-mediated endocytosis is inhibited by the coexpression of dominant-negative, GTPase-deficient K44A dynamin in AQP2-transfected LLC-PK1 cells (39). Wild-type dynamin is critically involved in the pinching off of clathrin-coated pits from the plasma membrane to produce clathrin-coated vesicles in the cytoplasm (15, 24, 33). Thus the steady-state location of AQP2 can shift from a predominant intracellular vesicle location to a plasma membrane location simply by inhibiting clathrin-mediated endocytosis. Because the effects of dominant-negative dynamin mutants can be examined only several hours after transfection into cells, these data provide little information about the rapidity of the constitutive AQP2 recycling process. A major aim of the present study was, therefore, to follow this process over a shorter time period (minutes), making use of a much more rapid method to inhibit endocytosis, i.e., membrane cholesterol depletion with methyl-β-cyclodextrin (mβCD) (30, 38).

A further unresolved issue is the role of phosphorylation in the trafficking and cell-surface accumulation of AQP2. Phosphorylation of AQP2 occurs at residue S256 in the COOH terminus and is certainly an essential event in the VP/cAMP-

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-mediated translocation pathway (12, 20). Kamsteeg et al. (17) concluded from oocyte expression studies that in each AQP2 tetramer (the functional form of AQP2), three of the four monomers must on average be phosphorylated to achieve cell-surface expression. Some nonphosphorylated AQP2 might, therefore, reach the cell surface by associating with phosphorylated AQP2 within each tetramer. However, while phosphorylation of AQP2 is necessary, it is not sufficient for regulated membrane insertion to occur because VP-induced calcium release from intracellular stores is also critically involved in AQP2 membrane accumulation (8). Despite these observations, a recent study concluded that nonphosphorylated AQP2 can reach the plasma membrane of cultured inner medullary collecting duct (IMCD) cells (42). These authors showed that okadaic acid, a serine/threonine phosphatase inhibitor, can induce AQP2 translocation to the plasma membrane in the presence of H-89, a protein kinase inhibitor, conditions under which the cytoplasmic S256 residue should not be phosphorylated. Taken together, these data imply that modification of either the target protein (AQP2) or elements of the trafficking machinery (e.g., actin, dynamin) can lead to protein accumulation on the cell surface.

Although phosphorylation of AQP2 is required for VP-induced membrane accumulation of AQP2, the idea that dephosphorylation is necessary for its subsequent endocytosis has not been supported by experimental data. Zelenina et al. (46) showed that dephosphorylation of the S256 residue of AQP2 does not seem to be required for endocytosis resulting from treatment of VP-stimulated cells with prostaglandin E2. This maneuver downregulates the VP response and reduces membrane water permeability. More recently, van Balkom et al. (43) showed that PMA, which also stimulates PKC activity, induces AQP2 endocytosis that is independent of the phosphorylation state of both the S256 residue as well as the S231 residue, which is a site of potential PKC-induced phosphorylation. Furthermore, phosphorylated AQP2 can also be found on intracellular vesicles (9). The latter result may, however, be expected because the phosphorylation event induced by VP/cAMP presumably occurs within the cell at the vesicular level before membrane accumulation of AQP2. The point in the recycling process at which dephosphorylation of the AQP2 S256 residue occurs is unclear.

A second goal of this study, therefore, was to determine the importance of phosphorylation in the cell-surface accumulation of AQP2 and, in particular, to determine whether interfering with the constitutive recycling pathway results in the cell-surface accumulation of AQP2 in the absence of VP-induced phosphorylation at residue S256. We show that inhibition of endocytosis results in a rapid (minutes) accumulation of AQP2 on the plasma membrane of LLC-PK1 and IMCD cells and that this membrane accumulation is independent of AQP2 phosphorylation at residue S256. This result may have important implications for achieving VP receptor (V2R)-independent delivery of AQP2 to the cell surface in patients with X-linked nephrogenic diabetes insipidus, whose VP signaling pathway is inoperative due to mutations in the V2R (25).

MATERIALS AND METHODS

Antibodies and chemicals. The hybridoma cell line 9E10 was purchased from American Type Culture Collection, and the cell culture supernatant containing anti-c-myc monoclonal antibody was used in this study. The anti-c-myc polyclonal antibody (A-14) and anti-dynamin-2 polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies (FITC- and CY3-conjugated) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Cell culture reagents, including Geneticin, DMEM, and FBS, were purchased from Gibco BRL (Grand Island, NY). Lysine VP and mIPCD were obtained from Sigma (St. Louis, MO). FITC-dextran (10,000 mol wt) was supplied by Molecular Probes (Eugene, OR).

cDNA constructs, adenovirus, and cell lines. The construct containing dynamin-2/K44A-GFP (pEGFP-dynamin-2/K44A) was provided by Dr. M. McNiven (Mayo Clinic and Foundation, Rochester, MN). The AdEasy adenovirus construction system was provided by Dr. T. He, Univ. Washington. To generate Ad-dynamin-2/K44A virus, the coding sequence of dynamin-2/K44A in the pEGFP-dynamin-2/K44A vector was amplified by PCR using Pfu DNA polymerase to introduce HindIII and EcoRV sites into the 5′- and 3′-regions of the coding sequence and to insert a flag tag to the NH2 terminus of the protein. The PCR fragment was subcloned to pShuttle-CMV at HindIII/EcoRV sites and the resultant construct was sequenced to confirm its predicted composition. pShuttle-CMV-dynamin-2/K44A and pAdEasy-1 were then cotransformed into Escherichia coli BJS183 to generate a recombinant viral plasmid by homologous recombination. HEK 293 cells were used as the packaging cells for large-scale virus production. The recombinant virus was purified by CsCl2 banding; the final yield was ~1012 viral particles per milliliter as estimated by OD260 measurement. The adenovirus was stored at −80°C in 10 mM Tris·HCl buffer, pH 8.0, containing 1 mM MgCl2 and 10% glycerol.

The preparation of stable LLC-PK1 cell lines expressing AQP2 with a c-myc tag at its COOH terminus (referred to as LLC-AQP2 cells in the remainder of the text) has been described previously (17). The production of LLC-PK1 cells stably expressing a phosphorylation-deficient S256A mutation of AQP2 has also been described previously (20). These cells are referred to as LLC-AQP2 (S256A) cells in the text. An identical approach was used to produce the AQP2-expressing mouse IMCD cell line (kindly provided by Dr. J. Schwartz, Boston Univ. Medical Center). These cells are referred to as IMCD-AQP2 cells throughout the text.

Adenovirus infection. The details of adenovirus infection of epithelial cells were described in our previous publication (39). Briefly, cells were grown on glass coverslips to 60% confluence, and the purified adenovirus Ad-dynamin-2/K44A was added to the culture medium at a ratio of 5 multiplicity of infection. The cells were then incubated with adenovirus for 16 h before use. We previously showed that infection of cells with adenovirus carrying the wild-type dynamin-1 has no effect on AQP2 distribution in LLC-PK1 cells nor on the response of the cells to VP (39). A similar lack of effect of wild-type dynamin-2 was found in the present study in initial data (not shown), but the control images shown in this report were from uninfected cells.

SDS-PAGE and immunoblotting analysis. Uninfected or Ad-dynamin-2/K44A-infected LLC-AQP2 cells were lysed in RIPA buffer and 12 μg of protein from each cell lysate were loaded onto an SDS-PAGE gel. Protein concentrations were determined with bicine-
molecular weights, bands were compared with prestained precision standards (Bio-Rad).

*Immunofluorescence staining: effect of dominant-negative dynamin expression on wild-type and S256A AQP2 localization.* LLC-AQP2, LLC-AQP2(S256A), or IMCD-AQP2 cells were grown on glass coverslips for immunofluorescence staining. To stimulate AQP2 accumulation on the plasma membrane, some cultures were incubated in 10 nM VP at 37°C for 15 min. Other cultures were infected with Ad-dynamin-2/K44A as previously described (39). Sixteen hours after infection, cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) at room temperature for 20 min. Cells were washed 3 × 5 min in PBS, permeabilized at room temperature with Triton X-100 (0.1%) for 4 min, incubated with 1% BSA in PBS for 15 min, and then with monoclonal anti-c-myc antibody for 1 h (to detect epitope-tagged AQP2). Anti-dynamin-2 antibody was used to detect dynamin-2 in LLC-AQP2 cells infected with Ad-dynamin-2/K44A. After being washed 3 × 5 min in PBS, coverslips were incubated for a further 1 h in secondary donkey anti-mouse IgG coupled to either FITC or CY3 (Jackson Labs). After final washes, coverslips were mounted on slides using Vectashield (Vector Labs, Burlingham, CA) diluted 1:1 with 1.5 M Tris-HCl (pH 8.9). The cells were examined using either a Nikon Eclipse 800 microscope or a Bio-Rad Radiance 2000 confocal microscope as previously described (39). All experiments were performed on at least three separate occasions, and data presented are representative of at least three independent sets of experiments in each condition.

*Immunofluorescence staining: effect of mβCD on wild-type and S256A AQP2 localization.* For these experiments, AQP2 trafficking was examined in LLC-AQP2, LLC-AQP2(S256A), and IMCD-AQP2 cells grown on Transwell filters. Approximately 1 × 10⁴ cells were suspended in 0.5 ml DMEM medium and seeded onto each 6.5-mm-diameter filter with a pore size of 0.4 µm (Transwell-Costar). Cells were incubated for 5 days to allow the formation of monolayers with 100% confluence. mβCD (10 mM) was added to the apical side of some filters and VP (10 nM) to the basalateral side of others. Cells were incubated with mβCD or VP for different time periods, from 5 to 30 min, as indicated in the legends for Figs. 1–6. Cells were fixed, permeabilized, and subjected to routine immunohistochemistry with monoclonal c-myc antibodies as above. Filter-grown cells were examined using a Bio-Rad Radiance confocal microscope.

*Quantification of FITC-dextran endocytosis by laser confocal microscopy.* LLC-AQP2 and LLC-AQP2(S256A) cells grown on coverslips were transferred into cell culture medium containing 5 µg/ml FITC-dextran immediately after acute cholesterol depletion (10 mM mβCD for 30 min) or without prior exposure to mβCD. They were incubated at 37°C for 15 min and then briefly washed six times in cold (4°C) PBS to remove cell surface FITC-dextran. Cells were then fixed for 20 min in 4% paraformaldehyde. To quantitate the uptake of the fluid-phase marker FITC-dextran, images of random areas of each coverslip were collected using a Bio-Rad Radiance 2000 confocal microscope with a ×40 objective. The sections to be quantified were taken from a focal plane that passed through the nucleus of the majority of cells in each random field. All images from different coverslips were collected with the same setting for laser intensity, pinhole aperture, photomultiplier gain, and offset. Using IP lab spectrum (Scanalytics, Vienna), a threshold value with yellow pseudocolor was applied to the images using the IP Lab “segmentation” feature so that the fluorescent endosomes were yellow and few or no highlighted pixels were located over the nucleus. Fluorescent regions consisting of three or fewer pixels, which were unlikely to represent endosomal structures due to their small size, were eliminated from the analysis using the exclusion criteria of the software. Data generated from each image gave a quantitation of the total area and average pixel intensity of the highlighted fluorescent structures. The product of area multiplied by intensity was used as a final measure of FITC-dextran uptake. FITC-dextran uptake in five to six images for each condition was measured in three independent experiments.

**Detection of cell-surface AQP2 by biotinylation.** LLC-AQP2 cells were grown to confluence in complete DMEM with 10% FBS. Cells were trypsinized with 0.5% trypsin-EDTA ( Gibco BRL) and collected through centrifugation. For each treatment, 5 × 10⁵ cells were resuspended in 1 ml DMEM with 10% FBS and incubated at 37°C for 20 min to allow recovery. VP (10⁻⁵ M) or mβCD (10 mM) was added to the cell suspension, mixed gently, and incubated at 37°C for a further 20 min. Then cells from each treatment were spun down and washed three times with cold PBS. The biotinylation agent sulfo-NHS-LC-Biotin (Pierce) was added to a final concentration of 1 mg/ml. Cells were incubated with biotin for 30 min at 4°C and then washed four times with cold PBS to remove any remaining biotinylation reagent. Cells were then lysed with RIPA buffer with protease inhibitors. The biotinylated cell lysates were precleared with protein A-gram Sepharose (Amersham) before immunoprecipitation. Two-hundred µg of cell lysate from each treatment were incubated with 1 µg of anti-c-myc polyclonal antibody (Santa Cruz Biotechnology) and 30 µl of protein A-Sepharose at 4°C for 2 h, followed by washing five times with RIPA buffer. The biotinylated, immunoprecipitated samples were resuspended in protein sample loading buffer with DTT and subjected to SDS-PAGE and Western blot analysis as previously described. Surface biotinylated AQP2 was visualized with streptavidin-HRP conjugate and total AQP2 in the cell lysates was detected using a previously characterized polyclonal antibody raised against an external epitope of AQP2.

**RESULTS**

**Adenovirus-mediated expression of dynamin-2/K44A in cultured epithelial cells.** The expression of endogenous dynamin-2 and adenovirus-mediated expression of dynamin-2/K44A were assessed by Western blot analysis. As shown in Fig. 1, endogenous dynamin-2 in LLC-AQP2 cells can be

![Fig. 1. Adenovirus-mediated expression of dynamin-2/K44A in LLC-aquaporin (AQP2) cells. The expression of endogenous dynamin-2 and adenovirus-mediated expression of dynamin-2 were qualitatively estimated by Western blot analysis with anti-dynamin-2 antibody. Left: endogenous dynamin-2 in LLC-AQP2 cells can be readily visualized at a molecular weight (MW) of 97. Right: however, adenovirus-mediated expression of the dynamin-2/K44A mutant results in a single band above 97. Interestingly, overexpressing the dynamin mutant suppresses the expression of endogenous dynamin-2 to an undetectable level in this Western blot. The slightly reduced mobility of the dynamin-2/K44A mutant is due to the additional NH₂-terminal flag tag sequence in this protein.**
readily detected with anti-dynamin-2 antibodies at a molecular weight of ~100,000. The dynamin-2/K44A mutant in infected cells appears as a strong protein band with a slight upward shift in molecular weight due to the presence of an NH2-terminal flag tag on this construct. Interestingly and unexpectedly, overexpression of this mutant dynamin seemed to suppress endogenous dynamin expression, as indicated by the almost complete disappearance of endogenous dynamin in Ad-dynamin mutant-infected cells (Fig. 1). Immunocytochemistry showed that ~95% of the cells exposed to the Ad-dynamin-2/K44A virus express dynamin-2/K44A protein, as we reported previously for adenoviral-mediated dynamin-1 expression (39). Furthermore, anti-flag monoclonal antibody staining of dynamin-2/K44A expressed in these cells colocalized with anti-dynamin-2 polyclonal antibody staining (data not shown).

In our previous study, the neuronal isoform dynamin-1 was expressed in epithelial cells using an adenoviral vector, but the epithelial isoform dynamin-2 was expressed in LLC-AQP2 cells by conventional transient transfection, yielding a relatively low transfection efficiency (39). The present data confirm that dynamin-2 can also be expressed with high efficiency in these cells using adenoviral-mediated infection.

Expression of dominant-negative dynamin-2/K44A causes plasma membrane accumulation of AQP2(S256A). In LLC-AQP2 cells, AQP2 was located mainly in a cytoplasmic vesicle pool under basal conditions (Fig. 2A), and it accumulated at the

![Fig. 2. Expression of dominant-negative dynamin-2/K44A results in phosphorylation-independent plasma membrane accumulation of AQP2. LLC-AQP2 (A, B, and C), inner medullary collecting duct (IMCD)-AQP2 (G, H, and I), as well as LLC-AQP2/S256A (D, E, and F) cells were treated with vasopressin (VP) for 10 min (B, E, and H) or infected with Ad-dynamin-2/K44A for 16 h (C, F, and I), respectively, before they were fixed and stained with anti-c-myc antibody. Both AQP2 and AQP2(S256A) were located in perinuclear vesicles in LLC-PK1 cells under baseline conditions (A, D, and G) as previously reported (20, 21). AQP2 relocated predominantly to the plasma membrane when cells were stimulated with VP (B and H), whereas the AQP2(S256A) mutant failed to translocate to the cell surface in response to VP (E) due to the mutation of its phosphorylation site (20). However, a marked membrane accumulation of both AQP2 and AQP2(S256A) occurred in cells infected with the dominant-negative dynamin-2/K44A mutant, indicating that this constitutive membrane accumulation is S256 phosphorylation independent.](http://ajprenal.physiology.org/)

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plasma membrane in response to VP stimulation (Fig. 2B). Membrane accumulation of AQP2 was also seen 16 h after infection with the dominant-negative dynamin-2 mutant in the absence of VP stimulation (Fig. 2C), as demonstrated previously (39). A similar result was obtained in another stably transfected cell line, IMCD-AQP2 cells, showing that the effect of the dynamin-2 mutation on membrane accumulation of AQP2 is not restricted to LLC-AQP2 cells (Fig. 2, H and I). In LLC-AQP2(S256A) cells, AQP2(S256) does not accumulate at the cell surface in response to VP stimulation, as previously shown (Fig. 2, D and E) (20). However, after infection with Ad-dynamin-2/K44A adenovirus for 16 h, AQP2(S256A) is mainly located in the plasma membrane (Fig. 2F) in a pattern that is indistinguishable from VP-treated LLC-AQP2 cells (Fig. 2B). This result suggests that constitutive recycling of AQP2 occurs in a S256 phosphorylation-independent pathway, whereas the VP-regulated recycling pathway is dependent on S256 phosphorylation.

**mβCD treatment inhibits endocytosis in LLC-PK1 cells.** mβCD is a cholesterol-depleting agent that reduces the rate of internalization of many membrane proteins and receptors, such as the transferrin receptor, without affecting intracellular receptor trafficking back to the cell surface (38). To determine the effect of acute cholesterol depletion on bulk fluid-phase endocytosis, LLC-AQP2 and LLC-AQP(S256A) cells were exposed to 10 mM mβCD for 30 min before they were transferred to labeling medium containing FITC-dextran, a fluorescent fluid-phase marker, for 15 min. FITC-dextran accumulates in endosomal vesicles, and the number and intensity of the labeled vesicles reflect the rate of fluid-phase endocytosis during the 15-min labeling time. Cells preincubated in normal medium showed substantial internalization of FITC-dextran into endosomes (Fig. 3A). In contrast, the uptake of FITC-dextran into mβCD-treated cells was significantly reduced (Fig. 3B). Quantitative analysis of confocal fluorescence microscope images showed that mβCD-treated cells internalized about five times less FITC-dextran than control cells (Fig. 3C). Acute cholesterol depletion inhibited fluid-phase endocytosis by both LLC-AQP2 and LLC-AQP2(S256) cells to the same extent (Fig. 3C).

**Membrane accumulation of AQP2 and AQP2(S256A) after acute cholesterol depletion with mβCD.** As demonstrated previously, membrane accumulation of AQP2 after blockade of clathrin-mediated endocytosis by Ad-dynamin-2/K44A occurs over 16 h (39). Shorter times of incubation with Ad-dynamin-2/K44A (less than 8 h) failed to show a detectable effect on membrane accumulation of AQP2 (data not shown). This probably represents the time taken for expression of the dominantly-negative dynamin mutant to functionally overcome the effect of endogenous, wild-type dynamin in these cells. To determine whether constitutive, VP-independent recycling occurs rapidly, mβCD treatment was used as a means of inhibiting endocytosis more acutely. As shown by the FITC-dextran data, the overall rate of endocytosis was reduced about fivefold after a 30-min exposure to this cholesterol-depleting drug. Cells were, therefore, treated with mβCD for 30 min and stained for AQP2. As shown in Fig. 4, B and F, mβCD treatment resulted in a marked accumulation of AQP2 on the plasma membrane of both LLC-AQP2 and IMCD-AQP2 cells. The IMCD cells grown on filters, however, displayed some plasma membrane staining even under baseline conditions.

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**Fig. 3.** Acute cholesterol depletion inhibit bulk fluid-phase endocytosis. LLC-AQP2 and LLC-AQP(S256A) cells were treated with 10 mM methyl-β-cyclodextrin (mβCD; CD) for 30 min to deplete membrane cholesterol before they were transferred to labeling medium containing 5 mg/ml FITC-dextran, a fluorescent fluid-phase marker. A: large number of FITC-labeled endosomes could be detected in cells preincubated in normal medium. B: in contrast, the uptake of FITC-dextran was significantly reduced in cells treated with mβCD. Quantitative analysis of confocal fluorescence microscopy images showed a significant difference in the uptake of FITC-dextran between the cholesterol-depleted cells and control cells. C: acute cholesterol depletion resulted in a 5-fold reduction in FITC-dextran endocytosis in LLC-AQP2 cells and LLC-AQP2(S256A) cells. Bar = 10 μm.
(Fig. 4E). In IMCD cells, the increase in plasma membrane staining after mβCD treatment was, therefore, less marked than in the LLC-PK₁ cells. However, the almost complete loss of intracellular vesicular staining caused by mβCD in IMCD cells was similar to the effect in LLC-PK₁ cells and presumably reflects vesicle (and AQP2) insertion into the plasma membrane. The reason for the higher baseline level of plasma membrane staining in IMCD cells compared with LLC-PK₁ cells is unknown, but a similarly high baseline membrane staining has also been reported in Madin-Darby canine kidney cells transfected with AQP2 (10). The membrane accumulation of AQP2 in both cell types can be seen clearly in confocal X-Z images. In LLC-AQP2(S256A) cells, AQP2/S256A also rapidly redistributed to the plasma membrane in response to mβCD treatment (Fig. 4D). These data show that inhibition of endocytosis with mβCD results in a marked membrane accumu-
mulation of AQP2 in both LLC-PK1 and IMCD cells and that this accumulation is independent of the phosphorylation state of the S256 residue of AQP2.

Time course of AQP2 membrane accumulation in response to VP and mβCD treatment. As shown in the previous experiment, membrane accumulation of AQP2 can occur rapidly after acute cholesterol depletion. To determine the relative speed of this accumulation in response to VP and mβCD, a time course study was conducted (Fig. 5). In VP-treated cells, membrane accumulation of AQP2 was seen as early as 5 min after treatment and became more obvious after 10–15 min. This basolateral membrane accumulation of AQP2 can be seen clearly in the confocal Z-series images. In cells treated with mβCD, membrane accumulation of AQP2 was detectable at the 10-min time point and became maximally developed between 15 and 30 min of treatment. The time frame of AQP2 membrane accumulation after mβCD treatment is somewhat slower than that seen in response to VP stimulation, probably due to the time required for the drug to reduce membrane cholesterol levels to a point at which endocytosis is inhibited. A similar time frame of rapid membrane accumulation of AQP2 was also observed in IMCD-AQP2 cells, as well as LLC-AQP2(S256A) cells (data not shown). These results show that rapid membrane accumulation of AQP2 caused by acute cholesterol depletion is a general phenomenon in these cell culture systems and that it is independent of S256 phosphorylation. This time course study indicates that membrane accumulation of AQP2 after acute cholesterol depletion can occur very rapidly, consistent with the hypothesis that AQP2 is constantly recycling through the plasma membrane in these cells.

Cell-surface biotinylation detects plasma membrane accumulation of AQP2 after mβCD and VP treatment. To confirm that the immunocytochemical data indeed reflect an increase in cell-surface accumulation of AQP2 after VP or mβCD treatment (rather than an intracellular shift of vesicles to a location close to the plasma membrane), surface expression (accumulation) of AQP2 was also assessed directly using cell-surface biotinylation. A similar technique was used previously by Fushimi et al. (12) to show cell-surface expression of AQP2 in transfected LLC-PK1 cells. Although some biotinylated AQP2 was present under baseline conditions in this assay, the amount of biotinylated AQP2 was clearly increased (2- to 3-fold by densitometry) after treatment with either VP or mβCD (Fig. 6), consistent with increased membrane accumulation of AQP2 under these conditions. The total amount of immunoprecipitated AQP2 in each of the samples was similar, as shown in Fig. 6B.

DISCUSSION

Collecting duct water permeability and urinary concentration are regulated by AQP2 trafficking, which occurs in response to an appropriate, usually hormonal (VP), stimulation of principal cells (3, 4, 11, 26, 31). This regulated AQP2 trafficking process is mediated via cAMP and, as demonstrated...
AQP2 was detectable in nontransfected LLC-PK1 cells (A). AQP2 in the control (lane 4) and treated LLC-AQP2 cells was similar, and no significant differences were found. The experiment was performed 3 times with similar results. Although some cell-surface AQP2 was detectable without stimulation, an increased amount of biotinylated AQP2 was clearly seen after treatment with VP and mβCD, consistent with increased membrane accumulation of AQP2 under these conditions. No immunoprecipitated biotinylated AQP2 was detected in nontransfected LLC-PK1 cells. The total amount of AQP2 in the control (panel) and treated LLC-AQP2 cells was similar, and no difference was observed. The experiment was repeated 3 times with similar results.

More recently, cGMP (2) is involved in other cell types. It involves complex signaling cascades that require phosphorylation and probably dephosphorylation of AQP2. Binding of VP to the V2R stimulates adenylyl cyclase, which increases intracellular cAMP levels, activates PKA, and subsequently activates PKA. Phosphorylation of AQP2 with a rapid time course that is similar to that of VP-induced pathway and direct blockade of endocytosis as a cause of AQP2 accumulation is that the latter does not depend on the state of S256 phosphorylation of AQP2. Both the phosphorylation-defective mutant AQP2(S256A) and wild-type AQP2 accumulated at the cell surface to an equal extent after treatment of cells with mβCD or infection with dominant-negative K44A dynamin to block clathrin-mediated endocytosis.

These data underline previous observations that AQP2 recycles constitutively between an intracellular pool of vesicles and the plasma membrane (13, 39) and demonstrate that this pathway occurs both in LLC-PK1 cells and in IMCD cells. Our previous data showed that AQP2 accumulated on the cell surface 16 h after expression of dominant-negative (K44A) dynamin to block endocytosis. This slow time course reflects experimental conditions in which the intracellular pool of newly expressed K44A dynamin must increase to a level sufficient to overcome the effect of endogenous wild-type dynamin on endocytosis. Others (1) have reported a similar time course for optimal blocking of endocytosis with this dynamin mutant. Our present data now show that cell-surface accumulation of AQP2 can be achieved within minutes after blocking endocytosis by cholesterol depletion with mβCD. This result indicates that AQP2 is recycling rapidly between the intracellular pool and the cell surface. Although mβCD has a major effect on endocytosis, an additional effect (either inhibitory or stimulatory) on exocytosis cannot be excluded completely. One report has shown that mβCD treatment inhibits dopamine release from PC-12 cells (7). In contrast, cholesterol depletion enhances calcium-stimulated exocytosis in mast cells while not affecting degranulation induced by antigen in the same cell type (34).

We recently provided direct evidence that AQP2 is internalized by a clathrin-mediated mechanism (13, 39), confirming circumstantial data that were obtained before the discovery of AQP2 (5, 6, 37). In this respect, AQP2 resembles many other recycling proteins, including the insulin-sensitive glucose transporter GLUT4. This protein behaves in a similar manner to AQP2 in that its plasma membrane accumulation and, therefore, its physiological function are stimulated by a hormone, insulin. GLUT4 also recycles constitutively between an internal vesicular pool and the cell surface (45). Its plasma membrane concentration can be increased by inhibiting endocytosis with K44A dynamin (1, 19, 29) and, as shown very recently, with mβCD (36). In the latter case, the membrane accumulation is also as rapid as with the physiological effector insulin in 3T3-L1 adipocytes. Nevertheless, it has been shown that insulin probably acts physiologically by increasing exocytosis (32, 45) at the same time as it inhibits endocytosis (16, 23). Therefore, both arms of the recycling pathway appear to be modified by insulin action. It should be emphasized that our present data do not rule out a similar dual effect of VP on both exocytosis and endocytosis of AQP2. Interestingly, while the end result of hormonal stimulation is similar for the GLUT4 and AQP2 proteins (i.e., accumulation on the plasma membrane), the second messenger cascades that result in their cell-surface accumulation are quite distinct. There is no evidence that cyclic nucleotides or PKA-induced phosphorylation are involved in the GLUT4 pathway. Instead, PI-3 kinase-stimulated phosphorylation of an insulin receptor substrate (IRS-1) plays a crucial role in the insulin response (18, 40).
Another interesting and unexplained difference between the two trafficking systems is that intact actin filaments are necessary for insulin-stimulated membrane accumulation of GLUT4 (28), whereas polymerization of actin has been reported to inhibit VP-induced AQP2 membrane accumulation (22).

It has been clearly shown that PKA-induced phosphorylation of AQP2 is necessary for its membrane insertion following VP treatment (12, 20). However, dephosphorylation of AQP2 does not seem to be necessary for its subsequent internalization. Prostaglandin E₂ stimulates removal of AQP2 from the surface of principal cells when added after VP treatment, without altering the phosphorylated state of AQP2 (46). Furthermore, PKC-mediated endocytosis of AQP2 is independent of the phosphorylation state of this water channel at residue serine-256 (43). Kamsteeg et al. (17) showed in Xenopus laevis oocytes that three out of the four AQP2 monomers in each functional tetramer need to be phosphorylated, on average, for AQP2 plasma membrane accumulation to occur in an oocyte expression system. In contrast, Valenti et al. (42) reported that okadaic acid induces AQP2 membrane insertion in cultured IMCD cells in the presence of the PKA inhibitor H-89. They concluded that okadaic acid stimulates the membrane translocation of AQP2 in a phosphorylation-independent manner. Our present data support the contention that AQP2 membrane accumulation can occur even when the S256 residue is not phosphorylated, because AQP2(S256A) accumulated at the cell surface when endocytosis was inhibited either by expression of dynamin K44A or after treatment of cells with mGluC. Furthermore, both wild-type and AQP2(S256A) accumulated rapidly at the plasma membrane after cholesterol depletion, indicating that they are both recycling rapidly in a constitutive pathway under baseline conditions.

As mentioned above, previous data show that phosphorylation of S256 is necessary for the VP-induced accumulation of AQP2 at the cell surface. There are, however, no data showing that phosphorylation of the S256 residue is required for AQP2 insertion into the plasma membrane; indeed, our present data indicate that phosphorylation of this residue is probably not required for plasma membrane insertion, at least in the constitutive pathway. If AQP2(S256A) recycles constitutively, then blocking endocytosis would necessarily result in its accumulation at the plasma membrane, the effect that is reported here. However, it remains possible that phosphorylation of AQP2 at S256 increases exocytosis, decreases endocytosis or both, to result in VP-induced accumulation at the cell surface. Our data do not allow us to distinguish among these possibilities. The conclusion from our present data is that blocking the clathrin-mediated endocytotic arm of the AQP2 recycling pathway is necessary for the VP-induced accumulation of AQP2 in transfected LLC-PK₁ and IMCD cells. The rapid AQP2 redistribution to the plasma membrane that occurs under these conditions suggests that caution must be exercised in interpreting the mechanism by which some experimental maneuvers cause plasma membrane AQP2 accumulation. More importantly, this finding (if it is found to extend to collecting duct principal cells in vivo) may have potential implications for achieving V2R- and phosphorylation (S256)-independent urinary concentration in X-linked nephrogenic diabetes insipidus patients. In the short term, cell culture models will allow for the rapid in vitro screening of compounds and drugs that have the desired effect of increasing cell-surface AQP2 in a receptor-independent manner. Identified reagents can then be tested in isolated, perfused tubules and kidneys and ultimately whole animals to determine their utility in the physiological setting. More studies will now be required to address the physiological effect of VP on the relative rates of exocytosis and endocytosis as a mechanism for inducing the cell-surface accumulation of AQP2 as well as to uncover the role of AQP2 S256 phosphorylation in the regulated recycling pathway.

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