Aquaporin-2 localization in clathrin-coated pits: inhibition of endocytosis by dominant-negative dynamin

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Aquaporin-2 localization in clathrin-coated pits: inhibition of endocytosis by dominant-negative dynamin. Am J Physiol Renal Physiol 282: F998–F1011, 2002. First published December 11, 2001; 10.1152/ajprenal.00257.2001.—Before the identification of aquaporin (AQP) proteins, vasopressin-regulated “water channels” were identified by freeze-fracture electron microscopy as aggregates or clusters of intramembrane particles (IMPs) on hormonally stimulated target cell membranes. In the kidney collecting duct, these IMP clusters were subsequently identified as possible sites of clathrin-coated pit formation on the plasma membrane, and a clathrin-mediated mechanism for internalization of vasopressin-sensitive water channels was suggested. Using an antibody raised against the extracellular C loop of AQP2, we now provide direct evidence that AQP2 is concentrated in clathrin-coated pits on the apical surface of collecting duct principal cells. Furthermore, by using a fracture-label technique applied to LLC-PK1 cells expressing an AQP2-c-myc construct, we show that AQP2 is located in IMP aggregates and is concentrated in shallow membrane invaginations on the surface of forskolin-stimulated cells. We also studied the functional role of clathrin-coated pits in AQP2 trafficking by using a GTPase-deficient dynamin mutation (K44A) to inhibit clathrin-mediated endocytosis. Immunofluorescence labeling and freeze-fracture electron microscopy showed that dominant-negative dynamin 1 and dominant 2 mutants prevent the release of clathrin-coated pits from the plasma membrane and induce an accumulation of AQP2 on the plasma membrane of AQP2-transfected cells. These data provide the first direct evidence that AQP2 is located in clathrin-coated pits and show that AQP2 recycles between the plasma membrane and intracellular vesicles via a dynamin-dependent endocytotic pathway. We propose that the IMP clusters previously associated with vasopressin action represent sites of clathrin-coated pit formation.

kidney epithelial cells; immunofluorescence; water channels; vasopressin stimulation; immunocytochemistry

AQUAPORINS (AQPs) are a family of transmembrane channel proteins that are present in diverse epithelial cell membranes and facilitate transepithelial water reabsorption and body fluid homeostasis (1, 19, 39, 40). AQP2 is the vasopressin (VP)-regulated water channel that is expressed in collecting duct principal cells in the kidney (9, 19), although this protein has been located in the testis and epithelial cells lining the vas deferens (47, 56) as well as in the inner ear (45a). Mutations in AQP2 protein result in autosomal nephrogenic diabetes insipidus, confirming the importance of this protein in urinary concentration (15, 34). Many studies have shown that AQP2 is moved from intracellular vesicles to the plasma membrane by hormonally induced, regulated trafficking of AQP2 (9), but in transfected cells, AQP2 also appears to cycle constitutively between the cell surface and intracellular vesicles (23).

Before the identification of AQPs, VP-sensitive “water channel” trafficking in the kidney was followed indirectly by freeze-fracture electron microscopy (EM) and by the use of endocytotic tracers such as horseradish peroxidase and FITC dextran (6). Freeze-fracture studies of VP target cells in amphibians and mammals revealed that aggregates or clusters of intramembraneous particles (IMPs) appeared on the plasma membranes of these cells only on hormonal stimulation of transepithelial water flow (13, 28, 33, 62). The area of cell surface occupied by these aggregates was directly correlated with the magnitude of the transepithelial water flow or urinary concentration in most instances (7, 32). Thus it was proposed that VP-sensitive water channels, now identified as AQP2 in mammals (20), may form clusters of IMPs when inserted into the plasma membrane. Subsequent studies revealed that these IMP clusters, which are probably sites at which water channels are aggregated in the plasma membrane, represent sites of clathrin-coated pit formation. Together with horseradish peroxidase tracer studies, this indirect evidence suggested that water channels were internalized by a clathrin-mediated pathway (10, 11, 58).

However, since the identification of AQP2 and the production of antibodies against the cytoplasmic COOH terminus of this protein, it has been difficult to...
confirm the clathrin-coated pit localization of AQP2 by immunogold EM. In previously published studies from several groups (including our own) that used immunogold EM, AQP2 was shown to be randomly distributed on the plasma membrane of principal cells, with no obvious accumulation in clathrin-coated structures (27, 31, 43, 48, 49, 52, 63). In the present study, we have used an antibody raised against an extracellular C-loop domain of AQP2 (4, 24) to reexamine the cell-surface localization of AQP2 in collecting duct principal cells. Furthermore, we used the fracture-label technique (17, 18) to localize cell-surface AQP2 in LLC-PK1 cells transfected with an AQP2-c-myc construct (LLC-AQP2 cells) (38).

We also examined AQP2 localization in LLC-AQP2 cells expressing GTpase-deficient, dominant-negative dynamin. It is well known that dynamin, a 100-kDa GTpase, plays an essential role in the release of nascent clathrin-coated pits from the plasma membrane during endocytosis (30, 45, 53). The precise role of dynamin in the membrane scission process is unclear, but several observations support the idea that dynamin has a mechanochemical function in vesicle release from the membrane (42, 55, 57, 59). Expression of GTpase-deficient, dominant-negative mutants of dynamin inhibits clathrin-mediated endocytosis in mammalian cells (12, 16, 21). This approach has proven to be a useful diagnostic tool for discriminating between dynamin-dependent and dynamin-independent mechanisms of endocytosis and for determining the role of dynamin-dependent endocytosis in a variety of cellular processes. Among the membrane proteins that internalize via a dynamin-dependent pathway are β2-adrenergic receptors (22, 64), GLUT4 (35, 36, 51, 61), the Na+/H+ exchanger NHE3 (14), and epithelial sodium channels (54). In addition, ARF6-mediated endocytosis in Madin-Darby canine kidney cells is dynamin dependent (2).

By using these approaches, we now provide direct evidence that AQP2 is located in clathrin-coated pits in collecting duct principal cells and that AQP2 internalization in LLC-AQP2 cells is a dynamin-dependent process.

**Materials and Methods**

**Peptides, antibodies, and chemicals.** Lysine VP was purchased from Sigma (St. Louis, MO). All cell culture reagents, including Genetin, DMEM, and fetal bovine serum were purchased from Gibco BRL (Grand Island, NY). The anti-c-myc monoclonal antibody (9E10) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-clathrin heavy chain monoclonal antibody was purchased from Transduction Laboratories (San Diego, CA). The anti-dynamin rabbit polyclonal antibody was purchased from Covance (Richmond, CA). The anti-dynamin 1 and anti-dynamin 2 rabbit polyclonal antibodies were from Affinity Bioreagents (Golden, CO). Secondary antibodies (FITC and CY3 conjugated) were purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

cDNA constructs, adenovirus, and cell lines. The recombinant adenovirus expressing the dynamin 1/K44A mutant was constructed by the method of Becker et al. (3) and was generously provided by Dr. Jeffrey Pessin (University of Iowa). HEK-293 cells were used as the packaging cells for large-scale virus production. The resultant virus was purified by CsCl banding; the final yield was ~10^12 viral particles, as estimated by measurements at an optical density of 260 nm. The adenovirus was stored at −80°C in 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM MgCl2 and 10% glycerol. The cDNA for the dynamin/K44A was generously provided by Dr. Sandra Schmid (Scripps Institute, La Jolla, CA). The constructs expressing dynamin 2/wt-green fluorescent protein (GFP), where wt is wild-type, and dynamin 2/K44A-GFP were provided by Dr. Mark McNiven (Mayo Clinic and Foundation, Rochester, MN) and prepared as previously described (36).

The preparation of a stable LLC-PK1 cell line 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 (38).

**Tissue fixation and immunogold detection of AQP2 in principal cells.** Three adult Sprague-Dawley rats were injected intraperitoneally with lysine VP (16 μg/kg body wt in 1 ml of 0.9% NaCl) 30 min before fixation of the kidneys by intravascular perfusion with paraformaldehyde lysine periodate fixative as previously described (41, 56). After a 5-min perfusion in situ, kidneys were removed and cut into 1- to 2-mm slices that were fixed for a further 6 h in paraformaldehyde lysine periodate buffer, pH 7.4. To examine AQP2 distribution in principal cells in the absence of VP stimulation, male Sprague-Dawley rats were anesthetized with pentobarbital sodium and the kidneys were briefly perfused via the abdominal aorta with Hanks’ balanced salt solution, pH 7.4, at 37°C equilibrated with 5% CO2-95% O2. When the kidneys were cleared of blood (~1 min), they were removed, and thin slices (~0.5 mm) were quickly cut with a Stadie-Riggs slicer (Thomas Scientific, Swedesboro, NJ). The slices were preincubated at 37°C for 15 min in equilibrated Hanks’ balanced salt solution as previously described. We have previously shown that this procedure results in VP washout and endocytosis of cell-surface AQP2 (4). For the detection of AQP2 in principal cells by EM, tissue slices were cut into thinner sections (50 μm) with a Vibratome (Ted Pella, Redding, CA). These final slices were incubated overnight with a previously characterized anti-AQP2 antibody (1:100 dilution) raised against an external epitope of AQP2 from the C-loop (C-GDlAVLNlHNnata) (24) without any detergent permeabilization step. This procedure ensured that only cell-surface (i.e., plasma membrane) AQP2 would be detected, as previously described (4). Some sections were incubated with preimmune rabbit serum or with antibody preabsorbed with the AQP2 peptide used for immunization as immunocytochemical controls. After being rinsed, the slices were incubated overnight in goat anti-rabbit IgG coupled to colloidal gold particles (10 nm diameter; Ted Pella). After a further rinsing, slices were fixed in 1% glutaraldehyde, postfixed in 2% OsO4 in distilled water for 2 h, dehydrated, and flat embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips CM10 electron microscope (Philips Electronics, Mahwah, NJ).

**Quantification of AQP2 gold particle labeling** was performed on 11 micrographs of cells taken from 1 animal. Images were taken at a final magnification of ×45,000. Total apical membrane length was measured in micrometers for each cell, and the length occupied by clathrin-coated pits was also measured. The number of gold particles in coated and noncoated membrane domains was then counted. All proce-
dures were performed with a Wacom graphics tablet attached to a Macintosh computer running the National Institutes of Health’s Image 4.1 software. The number of gold particles per micrometer of membrane was calculated for each membrane region, and the ratio of gold particle density per micrometer of membrane was obtained for the coated and noncoated domains.

Fracture labeling of LLC-AQP2 cells. LLC-AQP2 cells were incubated for 10 min with 10 μM forskolin followed by a 10-min incubation in medium alone to wash out forskolin in some cases. Control, forskolin-treated, and “washout” cells were cooled on ice while the medium was replaced with PBS containing 4% paraformaldehyde. Cells were kept on ice, and, after 15 min, the fixative solution was removed. Cells were incubated for 15 min with 50 mM NH₄Cl to arrest the fixation process, followed by subsequent incubations of 1 h each with 10, 20, and 30% glycerol in 0.1 M cacodylate buffer (pH 7.4). Fresh 30% glycerol buffer was added for overnight infiltration. Sheets of cells were scraped off the plastic support with a rubber policeman, placed on flat copper specimen holders, and rapidly frozen in N₂-cooled Freon 22. Frozen samples were transferred into a freeze-fracture apparatus (Cressington Scientific Instruments, Watford, UK). Sample temperature was raised to −145°C, and samples were cut under vacuum (10⁻⁷ Torr) to a thickness of ~0.1 mm. The specimen temperature was then raised to −120°C, and membranes were fractured with a final passage of the knife blade. Specimens were shadowed with 1.7-nm platinum at 45°, which was followed by a 6-nm carbon coat perpendicular to the fracture plane.

The technique described here, SDS-digested freeze-fracture replica labeling, was adapted from the procedure of Fujimoto (17, 18). Postfracture labeling with antibodies was performed after SDS digestion of replicas from lightly fixed cells. Replicas were thawed and floated on 2.5% SDS in PBS or 2.5% SDS in 0.1 M Na⁺/HCO₃⁻. Replicas that did not float were discarded. After shaking and prolonged incubation for 2 h, cleaned replicas were floated onto PBS containing 1% defatted and IgG-free bovine serum albumin (blocking solution) for 1 h. They were then floated for 2 h on anti-c-myc antibody (1:1,000) in blocking solution, washed three times for 15 min each with blocking solution, and incubated for 1 h on a drop of goat anti-mouse IgG-gold (15 nm diameter, 1:200 dilution). Replicas were washed in PBS, incubated with 2% glutaraldehyde in PBS for 2 min, and finally washed in distilled water for 2 min. The replicas, taken from two separate experiments, were examined on nickel grids with a Philips CM10 electron microscope.

Conventional freeze fracture of LLC-AQP2 cells expressing dynamin 1/K44A. LLC-AQP2 cells infected 16 h earlier with the dynamin 1/K44A adenovirus were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer. Cells were fixed by immersion for 1 h in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer. After cryoprotection for at least 1 h in 30% glycerol, cells were scraped from the culture dish, and clumps of cells were placed on a copper freeze-fracture support and frozen in Freon 22 cooled by liquid nitrogen. Freeze-fracture replicas were produced as described in Fracture labeling of LLC-AQP2 cells. After removal from the freeze-fracture device, the replicas were cleaned by immersion for 2 h in concentrated sodium hypochlorite bleach, washed three times for 5 min each with distilled water, picked up on copper EM grids, and examined with a Philips CM10 electron microscope. Random areas of plasma membrane from 20 control cells and 20 dynamin-transfected cells were photographed at a final magnification

Adenovirus infection. LLC-AQP2 cells were grown on 12 × 12-mm glass coverslips for 48 h to 60% confluence. Purified adenovirus Ad-dynamin 1/K44A was added to the culture medium directly at a ratio of five multiplicities of infection. The cells were incubated with adenovirus for 16 h before use. AQP2 trafficking in LLC-AQP2 cells and immunofluorescence detection. LLC-AQP2 cells or the transfectants on glass coverslips were incubated in DMEM without serum for 2 h before each experiment. To stimulate AQP2 movement to the plasma membrane, cells were incubated in 10 nM VP at 37°C for 10 min followed by fixation at room temperature for 20 min with 4% paraformaldehyde in PBS containing 5% sucrose. In the VP-washout experiments, the stimulated cells were washed with warm DMEM six times and incubated at 37°C for 90 min before fixation. After fixation, the cells were permeabilized at room temperature for 5 min with Triton X-100 (0.1%) for staining with anti-c-myc antibody (to detect epitope-tagged AQP2) or with SDS (1%), an antigen retrieval technique (8), for staining with the anti-clathrin antibody. Nonspecific antibody binding sites were blocked by a 10-min incubation with 1% BSA in PBS. The anti-c-myc monoclonal antibody was used to label AQP2 protein in cells, whereas the inherent fluorescence of the GFP tag was used to detect dynamic events occurring on LLC-AQP2 cells expressing the dynamin 2-GFP construct. Preliminary data that used an anti-dynamin 1 antibody showed that after adenoviral infection most of the cells in each culture expressed dynamin 1 (an isoform not normally detectable in nonneuronal cells). All primary and secondary antibodies were applied for 1 h at room temperature. Coverslips were mounted on slides with Vectashield (Vector Labs, Burling- ham, CA). The cells were examined with a Nikon Eclipse 800 microscope or a Bio-Rad Radiance 2000 confocal microscope. For final printing, the positive staining was pseudocolored in red or green, and images were printed with a Tektronix Phaser 440 dye sublimation printer. Transient transfections and adenovirus infections were both performed on three separate occasions, and data presented are representative of all three sets of experiments in each condition.

Thin-section EM of LLC-AQP2 cells expressing dynamin 1/K44A. LLC-AQP2 cells infected 16 h earlier with the dynamin 1/K44A adenovirus were fixed in 1% OsO₄, dehydrated in graded ethanol, and flat embedded in Epon 812. Thin sections were examined with a Philips CM10 electron microscope.

Conventional freeze fracture of LLC-AQP2 cells expressing dynamin 1/K44A. Conventional freeze fracture was performed on control cells and on cells expressing dominant-negative dynamin 1/K44A after adenoviral infection for 16 h. This method provides an en face view of large areas of plasma membrane on which images of membrane fusion and fission events can be visualized more readily than by thin-section EM. Cells were fixed by immersion for 1 h in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer. After cryoprotection for at least 1 h in 30% glycerol, cells were scraped from the culture dish, and clumps of cells were placed on a copper freeze-fracture support and frozen in Freon 22 cooled by liquid nitrogen. Freeze-fracture replicas were produced as described in Fracture labeling of LLC-AQP2 cells. After removal from the freeze-fracture device, the replicas were cleaned by immersion for 2 h in concentrated sodium hypochlorite bleach, washed three times for 5 min each with distilled water, picked up on copper EM grids, and examined with a Philips CM10 electron microscope. Random areas of plasma membrane from 20 control cells and 20 dynamin-transfected cells were photographed at a final magnification...
of ×21,000. The number of small circular membrane fusion sites, interpreted to reflect images of the necks of clathrin-coated pits, was counted and expressed as the number per square micrometer of membrane surface.

RESULTS

Coated pit localization of AQP2 in thin sections of collecting duct principal cells. In ultrathin sections of kidney collecting ducts from VP-treated rats, the apical plasma membranes of principal cells were heavily labeled with gold particles, representing sites of AQP2 antigenicity. As illustrated in Fig. 1, the majority of gold particles were positioned on the outermost surface of the plasma membrane of principal cells, which is consistent with the detection by this antibody of an externally oriented epitope of AQP2 as previously described (4). Although gold labeling in the form of clusters as well as individual gold particles was detected over microvilli and flat intermicrovillar regions of the membrane, many invaginated areas of the membrane were also heavily labeled. Most of these invaginated regions could be readily identified as clathrin-coated pits on the basis of the thick, electron-dense coat of clathrin that characterizes these structures in electron micrographs. The association of clathrin antigenicity with these coated domains on the plasma membrane of collecting duct principal cells has been described previously (11).

The appearance of the gold-labeled clathrin-coated membrane domains varied from relatively shallow invaginations to much more pronounced invaginations or so-called “Ω-figures” (Fig. 1, inset). Because no permeabilization step was included in the labeling procedure, intracellular vesicles containing AQP2 were not labeled in these sections, and some clathrin-coated vesicles that may have already detached from the cell surface were also unlabeled. Tissue slices that had been incubated in VP-free Hanks’ buffer for 15 min were also incubated with anti-AQP2 antibodies. As shown in Fig. 1B, very few gold particles were detectable on the apical plasma membrane of principal cells from these animals, indicating that under these VP-washout conditions, very little AQP2 is detectable at the cell surface. Furthermore, antibody labeling was not detectable on collecting duct intercalated cells that do not express AQP2 (4). Finally, no staining was seen in sections of tissue incubated in the absence of primary antibody or with antibody that was preincubated with the immunizing peptide.

Quantification of gold particle labeling in VP-treated rats revealed that, on average, the ratio of gold particle labeling per micrometer of membrane length in coated pits vs. noncoated apical membrane was 3.15 ± 0.73, showing a clear (>3-fold) enrichment of AQP2 in coated membrane domains. However, there was variability in the enrichment factor among different cells, ranging from no enrichment to an almost eightfold enrichment of AQP2 in coated pits, perhaps reflecting different activity states of the epithelial cell population. The total number of coated pits examined was 45, and the average membrane length of these pits was 0.27 ± 0.02 μm.

AQP2 localization in LLC-AQP2 cells by fracture labeling. Because the technique of fracture labeling is not easily adapted to whole tissues such as the kidney, we sought to examine the relationship between cell-surface AQP2 distribution and morphological features...
previously associated with water channel clusters (see the beginning of this study) in AQP2-transfected epithelial cells. Cells were first treated with forskolin (10 \( \mu \)M for 10 min) to induce the appearance of AQP2 on the basolateral cell surface, as previously described (38). Cells were then washed in buffer alone to stimulate the reinternalization of AQP2 by endocytosis, as previously described (37). Little or no gold label was found on the plasma membranes of nonstimulated cells (not shown). After forskolin stimulation, the amount of cell-surface labeling was increased, but the gold label was randomly dispersed on the basolateral membrane surface (4.8 gold particles/\( \mu \)m\(^2\); Fig. 2). After forskolin washout for 10 min, the AQP2 gold labeling became concentrated over membrane invaginations and IMP clusters (200 gold particles/\( \mu \)m\(^2\)), although randomly dispersed gold particles were still present on other areas of the membrane (4.1 gold particles/\( \mu \)m\(^2\); Fig. 3, A–C). These IMP clusters resembled the loose clusters of IMPs previously described on apical membranes of VP-stimulated principal cells (10, 25, 26). In addition to the gold-labeled clusters that were present in flat membrane domains (Fig. 3B), others were associated with deeper invaginations that are suggestive of endocytotic domains of the plasma membrane (81.3 gold particles/\( \mu \)m\(^2\); Fig. 3C). In this image, only 2.7 gold particles/\( \mu \)m\(^2\) were present on noninvaginated membrane domains.

Expression of dynamin 1 and 2 in LLC-AQP2 cells.

To show the functional role of dynamin in clathrin-mediated endocytosis, we overexpressed dynamin 1 and 2 mutants in LLC-AQP2 cells. The expression was verified by immunocytochemistry, as shown in Fig. 4. Dynamin 1, a neuronal specific isoform, is not endogenously expressed in LLC-PK\(_1\) cells. Staining of LLC-AQP2 cells with anti-dynamin 1 antibody showed no background (data not shown), whereas dynamin 1 virus-infected cells showed a strong positive staining (Fig. 4A). Dynamin 2, a ubiquitous isoform, is expressed endogenously in LLC-PK\(_1\) cells but at levels too low to be detected by immunocytochemistry (not shown). When cells were electroporated with a dynamin 2-GFP construct, \( \sim \)50% of the cells overexpressed dynamin 2-GFP (Fig. 4B). This overexpressed dynamin 2-GFP was also detectable in an identical pattern by using an antibody against the dynamin 2 COOH terminus (data not shown).

Expression of dynamin 1/K44A inhibits clathrin-coated pit detachment from the plasma membrane.

LLC-AQP2 cells were infected with the dynamin 1/K44A virus. They were treated with VP for 10 min and subsequent washout of VP for up to 90 min. Immunofluorescence labeling with anti-clathrin antibodies showed that clathrin was distributed mainly in a perinuclear and cytoplasmic location under baseline conditions, with little clear definition of the plasma membrane labeling (Fig. 5A). Expression of dynamin 1/K44A resulted in the appearance of a well-defined peripheral membrane pattern of staining in addition to intracellular staining (Fig. 5B). Membrane staining was also present after VP treatment (Fig. 5D) and VP washout (Fig. 5F). In the control cells, clathrin distribution was not clearly modified by VP stimulation and washout (Fig. 5, C and E).

The punctate, linear clathrin staining pattern in dynamin 1/K44A-expressing cells is suggestive of the accumulation of clathrin-coated pits that could not be released from the plasma membrane after the process.
of invagination. This was confirmed by conventional and freeze-fracture EM. In thin sections of dynamin 1/K44A-expressing cells, many images of invaginated coated pits with long narrow necks ~30–40 nm in diameter were found at the plasma membrane both by conventional EM (Fig. 6) and by freeze-fracture EM (Fig. 6, insets). Such images were rarely if ever found in noninfected cells. Freeze-fracture EM was performed to quantify this increase in cell-surface invaginations. Many small, circular depressions corresponding to the fractured necks of these “frozen” clathrin-coated pits were found in the dynamin 1/K44A-expressing cells (Fig. 7). The average diameter of these fractured necks (Fig. 7, inset) was 30.7 ± 6.8 nm (n = 28), and the number per square micrometer of membrane was 2.1 ± 0.4 (n = 11 cells) in the dynamin 1/K44A-expressing cells. Invaginations with similar morphology were not found in any of the 12 control cells that were examined. In cross fractures, vesicles attached to the plasma membrane by necks of various lengths could be detected (Fig. 6, inset). Similar structures were found on both the apical and the basolateral plasma membranes of the dynamin 1/K44A-expressing LLC-AQP2 cells.
Expression of dynamin 1/K44A inhibits AQP2 endocytosis. We previously demonstrated that AQP2 recycles between intracellular vesicles and the plasma membrane in response to hormonal stimulation and withdrawal (37, 38). As previously described, AQP2 translocated from a perinuclear vesicle pool (Fig. 8A) to the plasma membrane of LLC-AQP2 cells (Fig. 8C) on VP treatment (10 nM for 10 min), and it returned to intracellular vesicles 90 min after washout of VP (Fig. 8E). In contrast, AQP2 in dynamin 1/K44A-expressing cells showed a constitutive membrane location under baseline conditions (Fig. 8B), after VP treatment (Fig. 8D), and after VP washout (Fig. 8F).

Expression of dynamin 2/K44A also induces an accumulation of clathrin and AQP2 at the cell surface. The data obtained from dominant-negative mutant dynamin 1/K44A infection support the idea that dynamin plays a key role in AQP2 endocytosis and recycling from plasma membrane. However, dynamin 1 is an isoform of the protein that is restricted to neural tissue. Only dynamin 2, the ubiquitous isoform, is endogenously expressed in kidney epithelial cells. Thus we characterized the effect of dynamin 2 on clathrin localization and AQP2 endocytosis. We found that dynamin 2/K44A also inhibits AQP2 internalization, similar to the effect of the dynamin 1/K44A mutant on this process.

The constructs for dynamin 2 expression were tagged with GFP, a 30-kDa peptide, that potentially could affect the function of dynamin 2 in the endocytic process. Therefore, we transfected LLC-AQP2 cells with dynamin 2/wt-GFP to determine any potential effect of the GFP tag on dynamin 2 activity. In all of the figures showing dynamin 2 transfections, the effect of dynamin 2 on clathrin and AQP2 distribution can be easily seen by comparing their localization (red) in adjacent cells that either do or do not express the dynamin 2/wt-GFP construct (identified by its green fluorescence). The wild-type construct had no effect on clathrin localization (Fig. 9, A–C), whereas dynamin 2/K44A transfection resulted in an accumulation of clathrin on the cell surface (Fig. 9, D–F). Furthermore, wild-type dynamin transfection did not affect the intracellular localization of AQP2 (Fig. 10, A–C), and a normal VP-induced translocation of AQP2 to the cell surface was also seen in these transfected cells (Fig. 10, D–F). In contrast, dynamin 2/K44A transfection resulted in a cell-surface accumulation of AQP2 without VP stimulation (Fig. 11, A–C). Nontransfected cells retained a perinuclear distribution of AQP2. After VP stimulation, all cells showed the expected plasma membrane localization of AQP2 (Fig. 11, D–F).

DISCUSSION

The present data demonstrate that AQP2 can be associated with clathrin-coated pits in the apical plasma membrane of collecting duct principal cells, which supports earlier indirect evidence that AQP2 is internalized by a clathrin-mediated mechanism (10, 11, 58). The fracture-label data obtained by using LLC-AQP2 cells are also in agreement with this hypothesis, which is strongly supported by our finding that expression of dominant-negative, GTPase-deficient dynamin 1 and dynamin 2 in these cells causes accumulation of AQP2 on the cell surface secondary to an inhibition of endocytosis. In this model system, AQP2 was associated with membrane invaginations with the freeze-fracture characteristics of clathrin-coated pits (10, 46) after forskolin stimulation and washout. Furthermore, IMP clusters that resemble those induced by VP in principal cells, and which are proposed to be sites of AQP aggregation at the cell surface (6, 10, 11, 25, 26), were also labeled with gold particles in stimulated LLC-AQP2 cells. Thus our data support the hypothesis that VP-sensitive water channels are internalized by clathrin-mediated endocytosis and that IMP clusters.
are hallmarks of the membrane domains in which these water channels are concentrated before internalization. However, some IMP clusters were also detectable in nonstimulated LLC-AQP2 cells, where they were not labeled with gold particles (not shown). This indicates that although IMP clusters can contain AQP2, their appearance does not depend absolutely on the presence of AQP2 at the cell surface. This is consistent with the fact that clathrin-mediated endocytosis of many other membrane proteins occurs in these and other cells and that the generalized clustering of proteins destined for internalization in these regions results in IMP clusters that are detectable by freeze-fracture microscopy (46). This may indicate that not all of the individual IMPs that make up any given IMP cluster represent the AQP2 protein, although more direct evidence will be required to confirm this idea.

The label-fracture technique used in the present study was initially described by Fujimoto (17, 18), who made use of a rapid-freezing protocol for replicating and gold-labeling nonfixed cells. The use of fixed material was believed to preclude adequate cleaning of

![Fig. 5. Expression of dynamin 1/K44A (K44A/Dyn1) causes accumulation of clathrin at the plasma membrane. Nontransfected LLC-AQP2 cells and cells expressing dynamin 1/K44A were stimulated with vasopressin (VP; C and D) followed by VP washout (W/O; E and F). Cells were then fixed and labeled with anti-clathrin heavy chain monoclonal antibody. Compared with the mainly perinuclear localization of clathrin in control (CON) cells not expressing the dynamin mutant (A, C, and E), dynamin 1/K44A-expressing cells show a marked staining at the cell periphery under all conditions examined (B, D, and F). VP treatment did not result in a detectable increase in cell-surface expression of clathrin in control cells (C). Bar = 10 μm.](http://ajprenal.physiology.org/)

![Fig. 6. Electron microscopy showing the morphology of clathrin-coated pits in the plasma membrane of LLC-AQP2 cells expressing K44A dominant-negative dynamin 1. Use of tannic acid-saponin fixation (see MATERIALS AND METHODS) provides greater contrast and staining to the coated pits than conventional fixation and staining. Main panel: thin section of two invaginating coated pits (arrows) on the apical plasma membrane of the LLC-AQP2 cell. The pits have a rounded appearance, and in 1 case, the neck connecting the pit to the cell surface is clearly visible. Insets: 2 images of clathrin-coated pits seen by freeze-fracture electron microscopy. Right inset: invagination with a long, narrow neck. Bars = 0.5 μm (0.2 μm, insets).](http://ajprenal.physiology.org/)
Fig. 7. Freeze-fracture electron microscopy of dynamin 1/K44A-infected LLC-AQP2 cells. P-face of an apical membrane of a dynamin 1/K44A-expressing cell shows cross-fractured membrane projections (large oval structures) and many small IMPs. In addition, many small pits and depressions are present throughout the membrane P-face (arrows). These round, 30- to 40-nm-diameter depressions are shown at higher magnification in the inset and represent cross fractures through the necks of the many clathrin-coated pits that are “frozen” at the cell surface because of dynamin 1/K44A overexpression. These “neck” structures were virtually absent from control, nontransfected cells (see text for quantification). Bars = 0.3 μm (0.1 μm, inset).

Fig. 8. Expression of dynamin 1/K44A inhibits AQP2 endocytosis. LLC-AQP2 cells expressing dynamin 1/K44A (B, D, and F) as well as noninfected control cells (A, C, and E) were subjected to vasopressin (VP) stimulation and subsequent washout before they were fixed and stained with an anti-c-myc antibody. A: in the control cells, AQP2 is located in perinuclear vesicles under baseline conditions. It relocates predominantly to the plasma membrane after VP stimulation (C) and is retrieved to an intracellular compartment after VP washout (E). In dynamin 1/K44A-expressing cells, AQP2 is located on the plasma membrane under all three conditions (B, D, and F). Bar = 10 μm.
platinum-carbon replicas, which is necessary to allow labeling reagents to access the bottom surface of the replica, while preserving the antigenicity of attached proteins. The SDS treatment was used to digest away all biological material from the replica, with the exception of a very thin layer of protein that is in direct contact with the platinum. This thin, transparent layer of protein contains the antigenic sites that are detected by the subsequent antibody-gold-labeling procedure. We found, however, that brief fixation of LLC-AQP2 cells with 4% paraformaldehyde allowed preservation of antigenicity while not preventing the digestion of underlying cellular material. However, attempts to reproduce this procedure on the kidney have so far proven unsuccessful in our laboratory because of the difficulty in removing the thicker layer of biological material that remains attached to the replica after the fracturing procedure has been performed. Thus antibodies and gold reagents cannot gain access to the underside of the replica and cannot label the proteins that are present in the replicated membrane.

The reason that previous immunogold labeling procedures have failed to reveal a concentration of AQP2 in clathrin-coated pits at the cell surface is uncertain, but it could have two possible explanations. First, clathrin-coated domains are more difficult to positively identify in tissues that have been prepared for immunogold EM by embedding in hydrophilic resins such as Lowicryl K4M (11). Second, it is possible that antigenic sites on the COOH-terminal cytoplasmic tail of AQP2 are masked by interaction with clathrin and the accessory proteins present in these cell membrane domains. Because previously applied antibodies were raised against the extreme COOH terminus of the AQP2 protein, masking of this epitope would result in the negative findings. The use of an anti-external domain antibody in the present studies has now allowed the unequivocal localization of AQP2 in clathrin-coated pits in collecting duct principal cells. However, attempts to use this antibody to detect cell-surface AQP2 in LLC-AQP2 cells have so far proven unsuccessful for unknown reasons. The antibody was raised against a part of the external sequence of AQP2 that contains the

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Fig. 9. Expression of dynamin 2/K44A-GFP, but not dynamin 2/wt-GFP, where wt is wild-type, induces cell-surface accumulation of clathrin. LLC-AQP2 cells were transiently transfected with dynamin 2/wt-GFP (A–C) or dynamin 2/K44A-GFP (D–F) and stained for clathrin (red). A and D: green cells are dynamin 2-transfected cells, whereas those cells that appear dark are nontransfected. A–C: clathrin is located predominantly in a perinuclear and cytoplasmic pattern in both nontransfected cells and in the cells expressing dynamin 2/wt-GFP. D–F: in contrast, clathrin shifts to a punctate distribution along the plasma membrane of cells expressing dynamin 2/K44A-GFP (arrows, E). E and F: nontransfected cells surrounding the green transfected cells retain the normal perinuclear pattern of clathrin distribution.
N-glycosylation site, and it is possible that in LLC-AQP2 cells a cell-specific pattern of AQP2 glycosylation restricts antibody access to its binding site on the protein. Interestingly, the same antibody does recognize AQP2 in many intracellular vesicles in LLC-AQP2 cells (24). These data imply that the cell-surface form of this water channel is antigenically distinct from at least some of the intracellular protein in these transfected cells.

In support of our immunocytochemical data, the use of dominant-negative dynamin constructs demonstrated that AQP2 is internalized from the cell surface in a dynamin-sensitive pathway. Both dynamin 1/K44A (in adenovirus-infected cells) and dynamin 2/K44A (in transiently transfected cells) caused an accumulation of clathrin and AQP2 on the plasma membrane in the absence of VP or forskolin stimulation. VP induced no further cell-surface accumulation of AQP2 in K44A dynamin-overexpressing cells, and, after agonist washout, AQP2 remained on the cell surface. In control LLC-AQP2 cells, cell-surface AQP2 labeling was weak or absent under baseline conditions, greatly increased 10 min after VP stimulation, and was restored to a mainly intracellular location 90 min after agonist washout, as expected (37). However, no apparent increase in cell-surface staining for clathrin was obvious in VP-stimulated cells that were not expressing dominant-negative dynamin isoforms. However, we have previously shown that an increase in the number of cell-surface clathrin-coated pits can be quantified after VP stimulation of collecting duct principal cells (10). It is possible that the lower resolution afforded by immunofluorescence staining was not able to detect any VP-induced increase in plasma membrane-coated pits in the presence of a large amount of cytoplasmic staining. The more marked increase in plasma membrane-associated coated pits resulting from dynamin/K44A overexpression was, in contrast, readily detectable by immunofluorescence.

Taken together, our present data provide compelling evidence that AQP2 recycles constitutively between an intracellular pool and the cell surface, as previously proposed (23). This recycling pathway is blocked at the level of endocytosis from the cell surface on expression of dominant-negative dy-
namin, resulting in the observed accumulation even in the absence of agonist stimulation. Although dynamin has also been implicated in caveolin-mediated endocytosis (29, 50), we have found no morphological evidence that AQP2 is located in caveolae either in LLC-AQP2 cells or in principal cells (not shown). Indeed, we have previously reported that caveolin is a basolateral protein in principal cells (5), whereas AQP2 is predominantly apical in most regions of the kidney collecting duct. Thus our present data provide strong evidence to support the involvement of clathrin-mediated endocytosis in AQP2 recycling. These data also indicate that AQP2 accumulation at the cell surface can be a result of decreased endocytosis, at least under the relatively long (16 h) time scale of the present experiments. The relative importance of the rates of egress and endocytosis in the acute cell-surface accumulation of AQP2 that occurs in response to VP stimulation remains to be determined.

In summary, a role for clathrin-coated pits in water channel recycling was proposed in earlier studies on the basis of circumstantial evidence. We now provide direct immunocytochemical evidence in collecting duct principal cells in situ and freeze-fracture evidence in AQP2-expressing cells in culture that AQP2 is located in clathrin-coated pits and IMP aggregates, respectively. Furthermore, we show that AQP2 accumulates in the plasma membrane of LLC-AQP2 cells overexpressing dominant-negative dynamin 1 and dynamin 2. These data indicate that AQP2 internalization from the cell surface occurs by means of a clathrin-mediated endocytotic process. The data also demonstrate that AQP2 accumulation at the cell surface occurs after endocytosis is blocked by overexpression of GTPase-deficient dynamin isoforms in LLC-AQP2 cells, indicating that AQP2 constitutively recycles between an intracellular compartment and the plasma membrane in these cells.

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