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Constitutive and regulated membrane expression of aquaporin 1 and aquaporin 2 water channels in stably transfected LLC-PK_1 epithelial cells

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ABSTRACT **The aquaporins (AQPs) are a family of homologous water-channel proteins that can be inserted into epithelial cell plasma membranes either constitutively (AQP1) or by regulated exocytosis following vasopressin stimulation (AQP2). LLC-PK_1 porcine renal epithelial cells were stably transfected with cDNA encoding AQP2 (tagged with a C-terminal c-Myc epitope) or rat kidney AQP1 cDNA in an expression vector containing a cytomegalovirus promoter. Immunofluorescence staining revealed that AQP1 was mainly localized to the plasma membrane, whereas AQP2 was predominantly located on intracellular vesicles. After treatment with vasopressin or forskolin for 10 min, AQP2 was relocated to the plasma membrane, indicating that this relocation was induced by cAMP. The location of AQP1 did not change. The basal water permeability of AQP1-transfected cells was 2-fold greater than that of nontransfected cells, whereas the permeability of AQP2-transfected cells increased significantly only after vasopressin treatment. Endocytotic uptake of fluorescein isothiocyanate-coupled dextran was stimulated 6-fold by vasopressin in AQP2-transfected cells but was only slightly increased in wild-type or AQP1-transfected cells. This vasopressin-induced endocytosis was inhibited in low-K^+ medium, which selectively affects clathrin-mediated endocytosis. These water channel-transfected cells represent an *in vitro* system that will allow the detailed dissection of mechanisms involved in the processing, targeting, and trafficking of proteins via constitutive versus regulated intracellular transport pathways.**

The packaging, sorting, and selective delivery of proteins to the plasma membrane of all cells occurs via two distinct pathways, the so-called constitutive or nonregulated pathway and the regulated or stimulated pathway (1). Proteins that are constitutively inserted into the cell membrane usually show little or no intracellular accumulation, whereas vesicles that carry proteins along the regulated pathway often show a significant intracellular buildup as they await the appropriate physiological stimulus that results in their exocytotic fusion with the plasma membrane. There are many examples of proteins that are segregated between the two pathways, but a family of transmembrane water channels, the aquaporins (AQPs) (2), represents an especially intriguing group of proteins with which to dissect constitutive versus regulated intracellular targeting mechanisms.

The aquaporins are a family of homologous intrinsic membrane proteins that are responsible for the high water permeability of plasma membranes from a variety of cell types (2). CHIP28, now renamed AQP1, was the first to be identified and is the major erythrocyte water channel (3, 4). AQP1 was also cloned from rat kidney (5, 6), and antibody staining localized this protein to apical and basolateral plasma membranes of proximal tubules and thin descending limbs of Henle (7, 8). Several other transporting epithelial cell types and nonfenestrated endothelial cells also constitutively express AQP1 on their plasma membranes (2, 9).

In contrast, the homologous water channel AQP2 is expressed only in kidney collecting-duct principal cells (10, 11), and its membrane localization is tightly regulated by the antidiuretic hormone, vasopressin. In Brattleboro homozygous rats, which lack vasopressin and which have hypothalamic diabetes insipidus, AQP2 is located primarily on intracellular vesicles but is delivered to the apical plasma membrane by exocytosis following vasopressin treatment *in vivo* (12). A similar translocation was seen in isolated perfused tubules from normal rats (13). These data directly support the shuttle hypothesis of vasopressin action, which invokes a cycle of exo- and endocytosis of water channels to explain the stimulatory effect of vasopressin on collecting-duct water permeability (14–17).

Detailed investigations on the mechanisms underlying the selective sorting of AQP1 and AQP2 to constitutive versus regulated intracellular trafficking pathways, similar to those described in other cell types (18), have been hampered by the lack of a cell culture system in which these pathways for water channels are maintained *in vitro*. We previously described the expression of functional AQP1 in stably transfected Chinese hamster ovary cells (19), but expression of water channels in transfected epithelial cells has not been reported. The purpose of this study was to establish stably transfected epithelial cells expressing AQP1 and AQP2 in which constitutive versus regulated trafficking pathways to different plasma membrane domains could be investigated. LLC-PK_1 cells, a polarized epithelial cell line derived from pig kidney, were used because they are known to express the vasopressin V_2 receptor and to increase cAMP in response to vasopressin (20). Our results show that functional AQP1 and AQP2 are inserted into LLC-PK_1 cell plasma membranes via constitutive and regulated pathways, respectively, and that these cells represent an *in vitro* cell culture model in which the different trafficking and targeting pathways used by these proteins are retained.

MATERIALS AND METHODS

DNA Constructs. The coding sequence of rat AQP1 cDNA from plasmid pSP64.CHIP28k (6) was subcloned into pBluescript (Stratagene) at *Eco*RI and *Hin*dIII sites and then subcloned into the mammalian expression vector pcDNAI/

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Abbreviations: AQP, aquaporin; FITC, fluorescein isothiocyanate; TIR, total internal reflection.

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Neo (Invitrogen) at *Hin*dIII and *Xba* I sites. The cDNA encoding rat AQP2 in plasmid pSP64.WCH-CD, epitope-tagged with a 30-bp tail encoding a 10-aa c-Myc peptide (21), was subcloned into pBluescript at *Eco*RI and *Xba* I sites and then ligated into pcDNAI/Neo at *Xho* I and *Xba* I sites.

Cell Culture and Transfection. LLC-PK_1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, in a 5% CO_2 atmosphere at 37°C. LLC-PK_1 cells were plated at 15×10^4 per 60-mm dish 20 hr before transfection. For transfection, a DNA–calcium phosphate precipitate (0.5 ml) formed with 10 μg of plasmid DNA was added and the cells were incubated at 37°C for 18 hr, washed, and incubated further. After 14–20 days of selection in medium containing Geneticin (G418; GIBCO/BRL), resistant colonies were isolated with cloning rings and transferred to separate culture dishes for expansion and analysis.

Immunoblotting. Cells grown on a 100-mm dish were solubilized by heating at 65°C for 15 min in 1 ml of sample buffer [1% (wt/vol) SDS/30 mM Tris·HCl, pH 6.8/5% (vol/vol) 2-mercaptoethanol/12% (vol/vol) glycerol]. Proteins (150 μg per lane) were separated by standard Laemmli SDS/PAGE and transferred to Immobilon-P membrane (Millipore). For immunoblotting, membranes were incubated with AQP1 antiserum (7) diluted 1:1000 in blotting buffer or c-Myc monoclonal antibody (22) diluted 1:400. The membranes were washed and incubated with goat anti-rabbit or anti-mouse IgG (0.1 μg/ml) conjugated to horseradish peroxidase, and bands were detected by enhanced chemiluminescence (ECL; Amersham).

Immunofluorescence. Cells grown on glass coverslips for 3 days were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. The cells were washed in PBS, permeabilized with 0.1% Triton X-100 in PBS for 4 min, and incubated with anti-AQP1 serum (diluted 1:200 with PBS) or anti-c-Myc monoclonal antibody (diluted 1:40 with PBS) at room temperature for 1 hr. The cells were then incubated with Cy3-conjugated goat anti-rabbit or donkey anti-mouse IgG (Jackson ImmunoResearch) for 1 hr, washed, and mounted in Gelvatol before photography with a Nikon FXA photomicroscope.

Osmotic Water-Permeability Measurements. Water permeability (P_f) was measured by a real-time total internal reflection (TIR) microfluorimetric assay of osmotically induced cell volume changes (23). Cell monolayers were grown on 18-mm-diameter glass coverslips and the cytosolic compartment was fluorescently labeled by incubation with 10 μM calcein acetoxymethyl ester for 20 min. The coverslip was mounted in a perfusion chamber designed for TIR illumination by a Hg–Cd laser beam (λ = 442 nm). The TIR fluorescence excited in a thin layer of the cytosol (≈150 nm) adjacent to the cell–substrate interface was collected by a ×25 objective, filtered by a >515-nm long-pass filter, and detected by a photomultiplier tube. Because the number of cell-entrapped fluorophores is constant, relative TIR fluorescence is inversely proportional to cell volume. The ratio of cell volume change in response to rapid switching of perfusate osmolality was inferred from the TIR fluorescence time course. The time course for each experiment was fitted with a single exponential function; P_f was calculated from the exponential time constant, τ, by the relation $P_f = [\tau (A/V)_0 V_w \phi_0]^{-1}$, where $(A/V)_0$ is the initial cell surface/volume ratio measured by LLC-PK_1 cell shape reconstruction of serial confocal images, V_w is the partial molar volume of water (18 cm^3/mol), and ϕ_0 is the initial perfusate osmolality.

Quantitation of Vasopressin-Induced Endocytosis. A semiquantitative estimation of the extent of endocytosis of fluorescein isothiocyanate (FITC)-conjugated dextran under various conditions was obtained by laser confocal microscopy. Cells cultured on glass coverslips for 3 days were incubated with FITC-dextran (M_r 9400; Sigma) at 5 mg/ml either during vasopressin treatment (10 nM vasopressin for 10 min), for 10 min in the absence of vasopressin, or for 15 min after vasopressin washout (see below). The cells were then washed briefly and fixed with 4% paraformaldehyde for 20 min and washed three times with PBS. The coverslips were mounted in 50% glycerol in 0.2 M Tris·HCl (pH 8.0) containing 2% *n*-propyl gallate to retard quenching of the fluorescence signal. To quantitate the intensity of the fluorescent signal generated by internalized FITC-dextran, images of random areas of each coverslip were collected on a laser confocal microscope. The sections to be quantified were taken so that in the majority of cells, the subapical cytoplasm was visible (i.e., between the nucleus and the apical plasma membrane). Preliminary studies using a *z* series of sections taken at various levels throughout the cell monolayer showed that significant differences between treatments were detectable in all sections through the monolayer. All images were collected with the same values for photomultiplier gain, aperture diaphragm, and black levels. A threshold value with pseudocolor was then applied to the images so that the fluorescent endosomes were yellow, and histograms were generated for each image to quantitate surface area versus intensity of (yellow) pixels above the threshold value by using the Bio-Rad model MRC600 CM software. The pixel intensities of 10–15 images for each condition were measured.

K^+ depletion of some cells in combination with hypotonic shock (24) was carried out to inhibit clathrin-mediated endocytosis (24, 25). After a hypotonic shock for 5 min, cells were washed three times, incubated in K^+-free buffer for 15 min, and then treated for 10 min with 10 nM vasopressin in K^+-free buffer. Cells were washed rapidly in K^+-free buffer and reincubated for 15 min with FITC-dextran at 5 mg/ml in K^+-free buffer.

RESULTS

Clonal cell lines derived from stably transfected LLC-PK_1 cells were examined by Western blotting to confirm protein expression (Fig. 1). Wild-type cells showed no reactivity with the c-Myc antibody, as expected, but cells transfected with AQP2 cDNA expressed a protein at about 30 kDa that was heavily stained with the antibody against the c-Myc epitope. This size corresponds to native AQP2 (29 kDa) plus the attached 10-aa c-Myc epitope. LLC-PK_1 cells transfected with AQP1 cDNA showed a very heavy 28-kDa band and a broad 35- to 45-kDa band, representing nonglycosylated and glycosylated forms of the protein (3, 7). Staining was completely abolished after preabsorption of the AQP1 antibody with human erythrocyte membranes enriched in AQP1 by KI and detergent extraction of other proteins (data not shown). Wild-type LLC-PK_1 cells showed a very weak 28-kDa band with AQP1 antiserum, but no detectable immunofluorescence staining, indicating that they may contain a small amount of a crossreacting protein.

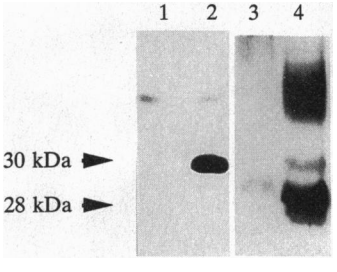

FIG. 1. Western blot showing staining of a 30-kDa band by c-Myc antibodies in AQP2-transfected (lane 2) but not wild-type LLC-PK_1 cells (lane 1). Wild-type LLC-PK_1 cells show weak staining of a 28-kDa band by AQP1 antibodies (lane 3) but cells transfected with AQP1 cDNA (lane 4) show very heavy staining of a 28-kDa band, as well as staining of a band at 35–45 kDa.

Expression and localization of water channels were examined by immunofluorescence. Wild-type LLC-PK_1 cells were not stained with AQP1 or c-Myc antibodies (data not shown). In AQP1-transfected cells, the protein was mainly localized at the plasma membrane and some intracellular staining (probably Golgi apparatus) close to the nucleus was also seen (Fig. 2*A*). In all cells, basolateral staining was detectable, but apical fluorescence was also present in many cells (data not shown). In contrast, AQP2 was predominantly localized to intracellular vesicles, although a minority of cells had weak plasma membrane staining (Fig. 2*B*).

The effect of vasopressin treatment on water-channel distribution was tested. After exposure of cells to 10 nM vasopressin for 10 min, the localization of AQP1 did not change, whereas AQP2 shifted from an intracellular vesicle pattern to a predominant plasma membrane staining (Fig. 2*C*). The staining was largely basolateral, with little detectable apical staining. After treatment with 10 μM forskolin for 10 min to increase intracellular cAMP, the redistribution of AQP2 to the plasma membrane was even more dramatic than with vasopressin (Fig. 2*D*); the localization of AQP1 was not altered by forskolin. These results indicate that the relocation of AQP2 from intracellular vesicles to the plasma membrane is regulated by vasopressin via an increase in intracellular cAMP. This effect of vasopressin was reversible; in prestimulated cells, washout of vasopressin for 30 min caused a partial relocation of immunostaining to vesicles, and after 60 min, all the staining was in cytoplasmic vesicles as in Fig. 2*B*, with little fluorescence detectable on the plasma membrane (data not shown).

To examine whether these observations reflected the presence and redistribution of functional AQP1 and AQP2 in stably transfected cells, osmotic water permeability was measured by TIR fluorescence of calcein-labeled cells. This technique was used because bulk transepithelial water flow cannot be measured in cells grown on coverslips. Fig. 3*A* shows the time course of TIR fluorescence in AQP2-expressing cells in response to a 100-mOsm outwardly directed NaCl gradient. The osmotic gradient caused water influx, cell swelling, and a decrease in TIR fluorescence. The rate of the swelling response was remarkably increased after vasopressin treatment. Averaged P_f values are summarized in Fig. 3*B*. In wild-type LLC-PK_1 cells, P_f was not altered by $HgCl_2$ or vasopressin. In AQP1 transfected cells, P_f was high and was strongly inhibited by $HgCl_2$. Treatment with 10 nM vasopressin had no effect on osmotic water permeability in AQP1-transfected cells. In contrast, P_f in the AQP2-expressing cells was increased significantly by vasopressin treatment and the increase in P_f was inhibited by $HgCl_2$. These results support the conclusion that vasopressin treatment results in the recruitment of functional water channels into the plasma membrane of AQP2-transfected LLC-PK_1 cells and that, in contrast, AQP1-transfected cells have a constitutively high water permeability that is not regulated by vasopressin.

Previous work in vasopressin-sensitive epithelia has shown that vasopressin treatment and washout stimulates a process of water-channel recycling, involving an increase in exo- and endocytotic events (26–29). To examine whether this feature of vasopressin action was present in AQP2-transfected cells, cultures were incubated with 10 nM vasopressin for 10 min, rapidly washed in PBS, incubated with FITC-dextran at 5 mg/ml for a further 15 min, then rapidly washed again, fixed, and examined with a laser confocal microscope. FITC-dextran

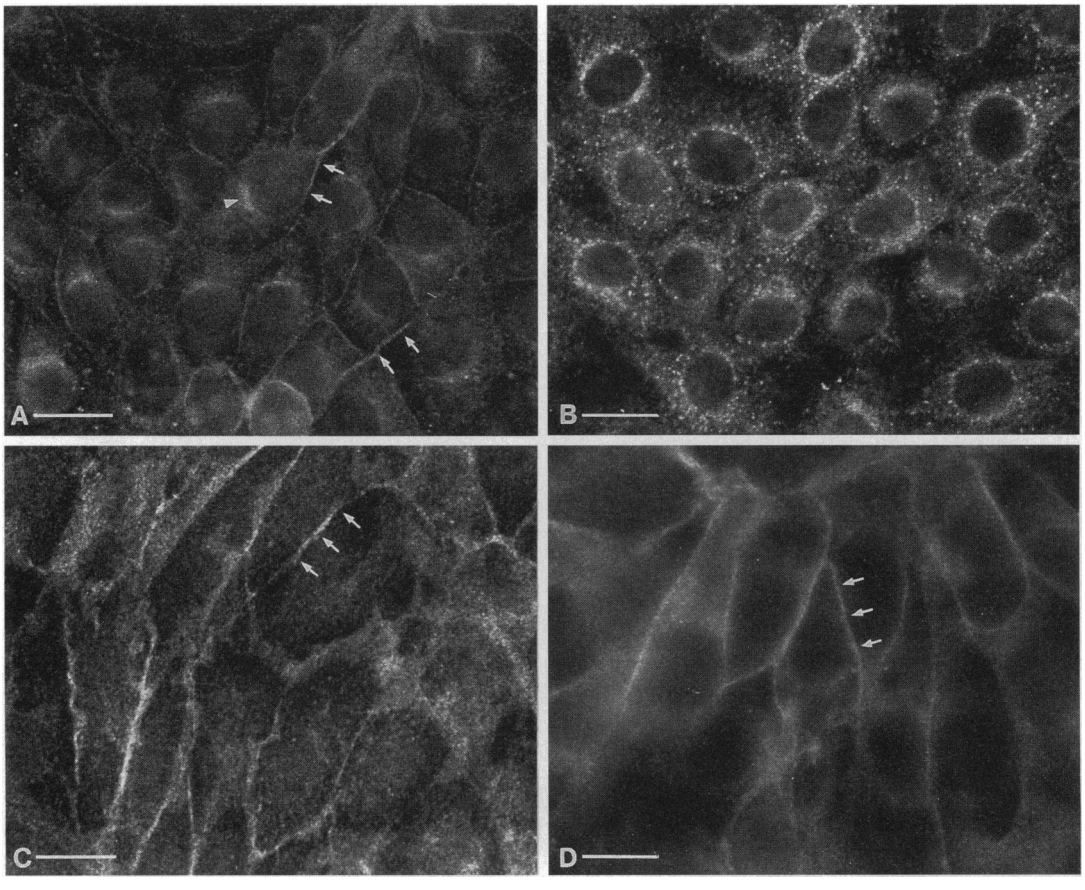

FIG. 2. Immunofluorescence localization of AQP1 in transfected LLC-PK_1 cells (*A*). The protein is located on the plasma membrane (arrows) as well as on an intracellular perinuclear structure, probably the Golgi apparatus (arrowhead). In AQP2-transfected cells (*B*), the c-Myc antibody stains many cytoplasmic vesicles, but the plasma membrane of most cells is unstained under basal conditions. AQP2 shows a plasma membrane localization (arrows) after vasopressin (*C*) or forskolin (*D*) treatment. (Bar = 20 μm.)

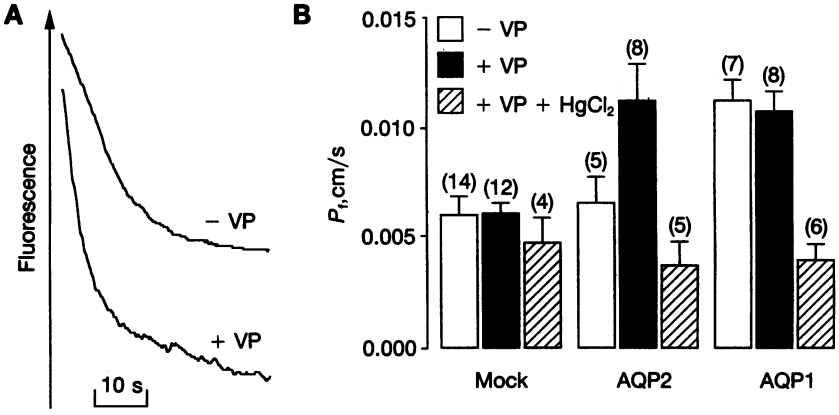

FIG. 3. Vasopressin increases the plasma membrane water permeability in AQP2-transfected LLC-PK_1 cells. (*A*) Representative TIR fluorescence time course of AQP2-transfected cells at 10°C in response to a 100-mOsm inwardly directed NaCl gradient. Where indicated (+VP), vasopressin (100 microunits/ml) added to the cell medium for 5 min at 37°C. (*B*) Averaged (±SEM) osmotic water permeability coefficients, P_f, of wild-type (mock-transfected) and AQP2- or AQP1-transfected cells before incubation with vasopressin (−VP), after vasopressin (+VP), and after incubation with VP plus 300 μM $HgCl_2$.

endocytosis in AQP2-transfected cells was markedly stimulated (almost 6-fold) after vasopressin treatment (Fig. 4). In contrast, incubation with the hormone caused only a slight increase of FITC-dextran endocytosis in wild type and AQP1-transfected cells. Hormone washout was not necessary to induce endocytosis, because a similar increase of FITC-dextran endocytosis was also measured in AQP2-transfected cells treated for 10 min with vasopressin in the continual presence of FITC-dextran (data not shown).

In collecting-duct principal cells, water channels are removed from the cell surface by clathrin-mediated endocytosis (26–29). To determine whether the observed increase in endocytosis in vasopressin-stimulated, AQP2-transfected cells might be clathrin-mediated, we examined the effect of K^+ depletion, a maneuver that selectively inhibits clathrin-mediated endocytosis but does not affect other endocytotic events (24, 25). Vasopressin failed to increase FITC-dextran endocytosis in K^+-depleted cells (Fig. 5), consistent with an involvement of clathrin in this process.

DISCUSSION

LLC-PK_1 epithelial cells were stably transfected with cDNAs encoding AQP1 and AQP2. The AQP1 protein was expressed on the plasma membrane in a constitutive fashion, and the AQP1-transfected cells had a significantly higher basal water permeability than either wild-type or AQP2-transfected cells. In contrast, AQP2 in transfected cells was located mainly on a population of intracellular vesicles but was recruited to the plasma membrane after vasopressin treatment or after an increase in intracellular cAMP by forskolin. This recruitment was accompanied by a significant increase in the mercurial-sensitive water permeability. Thus, the behavior of these two AQPs when transfected into LLC-PK_1 epithelial cells indicates that they are sorted into distinct secretory pathways that allow constitutive (AQP1) versus regulated (AQP2) insertion into the plasma membrane. Remarkably, their behavior in transfected cells recapitulates the secretory pathways used by these proteins to reach the plasma membrane *in vivo*; these transfected cells, therefore, represent an *in vitro* system which will allow the detailed dissection of mechanisms involved in the processing, targeting, and trafficking of homologous proteins by these distinct intracellular pathways.

Our observations suggest that both AQP1 and AQP2 contain sorting information that is sufficient to direct their packaging into constitutive versus regulated transporting vesicles. Some studies have already shown that a variety of amino acid changes in AQP2 modify water channel activity in a *Xenopus* oocyte expression system, but it is not known whether these proteins are actually delivered to the plasma membrane (30). Conversely, some natural mutations in human AQP2 that result in nephrogenic diabetes insipidus (31) cause retention of the AQP in the rough endoplasmic reticulum (32), but the water-channel activity of these proteins is not known.

Preliminary studies showed that both immunolocalization and immunoblotting of the native AQP2 protein transiently expressed in LLC-PK_1 cells was weak with an antibody raised against a peptide containing the C-terminal 15 aa of AQP2. The reason for this finding is unknown, but in the present studies, the AQP2 molecule was, therefore, modified by the addition of a c-Myc epitope tag on the extreme C terminus to allow efficient immunodetection with c-Myc monoclonal an-

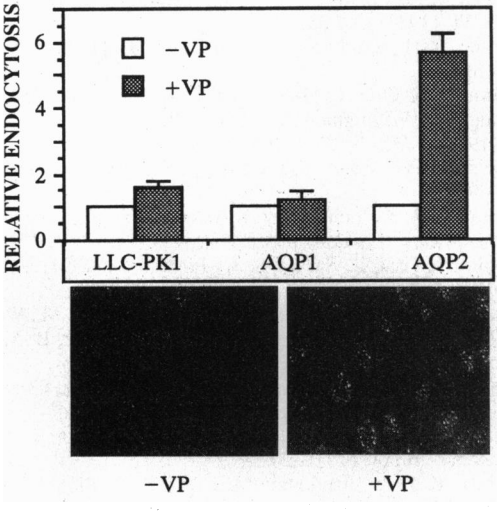

FIG. 4. (*Upper*) Semiquantitative analysis of FITC-dextran endocytosis in wild-type LLC-PK_1 cells, AQP1-transfected cells, and AQP2-transfected cells by laser confocal microscopy. The low basal uptake of FITC-dextran (−VP) was not significantly increased by vasopressin (+VP) in either wild-type cells or AQP1-transfected cells but was increased about 6-fold in AQP2-transfected cells. (*Lower*) Representative confocal images of basal FITC uptake and the increased uptake in vasopressin-treated AQP2-transfected cells.

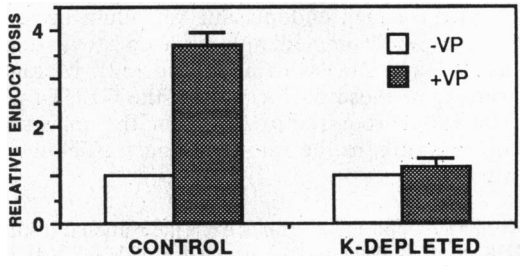

FIG. 5. Semiquantitative analysis of vasopressin-stimulated endocytosis of FITC-dextran in AQP2-transfected cells in normal medium and after hypotonic shock and incubation in K^+-depleted medium. The vasopressin-induced increase in AQP2-transfected cells in normal medium was completely inhibited by K^+ depletion.

tibodies. Despite this C-terminal modification, AQP2 was translocated from an intracellular site to the plasma membrane, an effect similar to that expected from work on collecting-duct principal cells *in vivo* (12) and in isolated perfused tubules (13). However, immunolocalization studies showed that after stimulation, AQP2 was concentrated in the basolateral plasma membrane of LLC-PK_1 cells rather than the apical location predicted from some previous studies. There are several potential explanations for this finding: (*i*) the c-Myc tag might affect targeting; (*ii*) the targeting of some proteins in transfected cells is variable and cell-specific [e.g., chimeric Na^+/K^+-ATPase–H^+/K^+-ATPase molecules are inserted into different membrane domains in LLC-PK_1 and MDCK cells (33)]; (*iii*) maximum functional polarity might not be established in cells grown on coverslips. Further work is needed to address these possibilities. However, some basolateral staining for AQP2 has been detected in collecting-duct principal cells by Nielsen *et al.* (11). In contrast to AQP2, AQP1 was constitutively delivered to both apical and basolateral plasma membrane domains in LLC-PK_1 cells, as it is in kidney proximal tubules and thin-descending-limb epithelial cells (7, 8).

The dramatic increase in endocytotic activity of LLC-PK_1 cells transfected with AQP2 indicates that this molecule also contains information that stimulates membrane internalization. The plasma membrane localization of AQP2 was completely reversible after vasopressin washout, indicating that water channels can be reinternalized as proposed by the shuttle hypothesis of vasopressin action (16, 17, 27–29). Because no experiments were performed in the presence of protein-synthesis inhibitors, we cannot rule out the possibility that the AQP2 appearing on intracellular vesicles during vasopressin washout was newly synthesized. Colocalization of FITC-dextran and AQP2 in the same vesicles was technically difficult in intact cells because the permeabilization step required for AQP2 detection caused a complete release of the internalized fluorophore from endosomes.

This vasopressin (or cAMP)-regulated endocytotic pathway appears to be virtually absent from wild-type or AQP1-transfected cells, eliminating the possibility that AQP2 is simply a passenger in vesicles that are already part of an endogenous stimulated pathway. Transfection of cells with AQP2 seems, therefore, to induce the appearance of a novel regulated transport vesicle that cannot be detected in wild-type cells. In this respect, the behavior of AQP2 resembles that of the GLUT4 glucose transporter, which contains discrete targeting sequences for endocytosis in its C terminus. Interestingly, dileucine internalization sequences that are critical for endocytosis of the GLUT4 protein (34, 35) are also present in AQP2, but not in AQP1. In addition, both GLUT4 and AQP2 are internalized by clathrin-coated pits (27, 28, 36). However, as currently mapped by hydropathy analysis, the SLL motifs have been allocated a position in the last transmembrane domain of AQP2 (10). Interestingly, one of the naturally occurring mutations in AQP2 that results in retention in the rough endoplasmic reticulum involves replacement of Ser^{216} immediately adjacent to a dileucine motif (aa 217 and 218) with a proline (32). Mutagenesis studies similar to those performed for the GLUT4 glucose transporter will be required to determine the importance of this or other motifs in the targeting and trafficking of the aquaporins.

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