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Internalization of vasopressin analogs in kidney and smooth muscle cells: Evidence for receptor-mediated endocytosis in cells with V_2 or V_1 receptors

(fluorescence microscopy/water movement/blood pressure)

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ABSTRACT To determine whether receptor-mediated endocytosis occurs in vasopressin-responsive cells, we developed a model system using synthetic fluorescent-labeled vasopressin analogs and A10 (smooth muscle) and LLC-PK_1 (kidney epithelial) cells in culture; these cell lines express V_1 and V_2 vasopressin cell surface receptor types, respectively. We used epifluorescence microscopy to examine the binding, internalization, and intracellular destination of [1-(2-mercapto)propionic acid,8-lysine-N^6-carboxytetramethylrhodamine]vasopressin (R-MLVP) and [1-(2-mercapto)propionic acid,8-lysine-N^6-carboxyfluorescein]vasopressin (F-MLVP) in these cells. The rhodamine-labeled fluorescent vasopressin analog, R-MLVP, initially bound in a diffuse manner at the cell surface of both A10 and LLC-PK_1 cells and could be displaced by excess unlabeled [8-arginine]vasopressin. After incubation at 37°C, bound ligand rapidly aggregated into small clusters or patches, which were internalized in a manner consistent with receptor-mediated endocytosis. Subsequent processing of internalized ligand–receptor complexes appeared to differ between A10 and LLC-PK_1 cells. In the case of LLC-PK_1 cells, ligand was delivered to a tightly focused lysosome compartment in the perinuclear region of the cell, and receptor molecules were replenished at the cell surface. The lysosomal location of ligand was supported by the quenching of fluorescence in the internalized vesicles when F-MLVP was used as a fluorescent tracer. In the case of A10 cells, ligand became localized to a vesicular compartment and reappearance of receptor at the cell surface was limited. Our data are consistent with the occurrence of receptor-mediated endocytosis of vasopressin in cells with V_1 and V_2 receptors.

Peptide hormones interact with cell surface receptors and stimulate the formation of second messengers that can elicit a variety of biological responses. In many systems, binding of peptide hormones, effector ligands such as growth factors, and nutrients such as cholesterol and iron (coupled to the appropriate carriers) to their receptors is accompanied by internalization of the ligand–receptor complex along the receptor-mediated endocytosis pathway (1). The role of receptor-mediated endocytosis has been clearly established for low density lipoprotein and transferrin (2–5). In these cases, the ligands are internalized into cells, and cholesterol and iron enter into the cellular metabolic pools in a physiologically controlled manner. The precise role of receptor-mediated endocytosis and other modes of ligand internalization in the physiological effects of peptide hormones, however, is less clear and is being actively investigated.

To determine whether receptor endocytosis occurs in vasopressin-sensitive cells, we established a model system using fluorescent-labeled vasopressin analogs and cells that express specific vasopressin receptor types. Vasopressin, a cyclic nonapeptide hormone, elicits a variety of biological responses by binding to cell surface receptors. Vasopressin is vital for the control of urine concentration and it plays a role in the maintenance of blood pressure in animals; additionally, it increases glycogenolysis in hepatic cells (6–8). Two types of vasopressin receptors (V_1 and V_2) have been distinguished based on the types of second messengers they elicit and upon their relative affinities for various vasopressin analogs (9). The binding of vasopressin to V_1 receptors, which are present in liver, smooth muscle cells, and platelets, results in the formation of inositol phosphates and diacylglycerol, which increase intracellular Ca^{2+} and protein kinase C activity. The interaction of vasopressin with V_2 receptors, such as those found in the collecting-duct cells of the kidney, stimulates adenylate cyclase activity, thereby increasing the intracellular concentrations of adenosine 3′,5′-(cyclic)monophosphate (cAMP). As the numbers of hormone-specific receptors on the surface of a cell determine the cell's response to that hormone, we attempted to determine whether vasopressin cell surface receptors were internalized and thus made unavailable for binding by the process of receptor endocytosis. We used two established cell lines of vascular and renal origin that respond to vasopressin via V_1 and V_2 vasopressin receptor types, respectively. LLC-PK_1 (ATCC no. CL 101), an established cell line from pig kidney, maintains the characteristics of a polar epithelial cell in culture and responds to vasopressin by increasing intracellular cAMP content (10–12). A10 (ATCC no. CRL 1476), an established smooth muscle cell line from rat embryonic thoracic aorta, responds to vasopressin by increasing phosphatidylinositol turnover and intracellular Ca^{2+} content (13–15). We used epifluorescence microscopy to examine the binding, uptake, and intracellular destination of fluorescent-labeled vasopressin analogs, [1-(2-mercapto)propionic acid,8-lysine-N^6-carboxytetramethylrhodamine]vasopressin (R-MLVP) and [1-(2-mercapto)propionic acid,8-lysine-N^6-carboxyfluorescein]vasopressin (F-MLVP), in LLC-PK_1 and A10 cells.

MATERIALS AND METHODS

LLC-PK_1 cells were grown as a monolayer in 75-cm^2 polystyrene culture flasks (Corning) containing medium 199 (GIBCO) supplemented with 3% fetal bovine serum plus penicillin (100 units/ml) and streptomycin (100 μg/ml). A10 cells were grown as a monolayer in 75-cm^2 polystyrene

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Abbreviations: R-MLVP, [1-(2-mercapto)propionic acid,8-lysine-N^6-carboxytetramethylrhodamine]vasopressin; F-MLVP, [1-(2-mercapto)propionic acid,8-lysine-N^6-carboxyfluorescein]vasopressin.

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culture flasks containing Dulbecco's modified Eagle's medium (GIBCO) supplemented with 20% fetal bovine serum plus penicillin and streptomycin. Confluent cells were subcultured by treating with a solution of 0.25% trypsin and 0.25% EDTA in phosphate-buffered saline.

The fluorescent vasopressin analogs R-MLVP and F-MLVP were prepared, purified, and characterized as described (16). In brief, R-MLVP and F-MLVP were synthesized by coupling carboxytetramethylrhodamine succinimidyl ester and carboxyfluorescein succinimidyl ester (Molecular Probes), respectively, to the 6-amino group of the lysine residue in [1-(2-mercapto)propionic acid,8-lysine]vasopressin. R-MLVP and F-MLVP were purified by reverse-phase high-performance liquid chromatography and their structures were confirmed by fast-atom bombardment mass spectrometry. The affinity of R-MLVP and F-MLVP for vasopressin receptors in LLC-PK_1 cells, A10 cells, and canine renal plasma membranes was determined by a competition binding assay.

LLC-PK_1 and A10 cells to be used for fluorescence microscopy experiments were cultured in monolayers on circular glass microscope coverslips (12 mm in diameter; Fischer Scientific) in 24-well culture trays. The growth medium was removed and the cells were rinsed twice with 0.5 ml of ice-cold phosphate-buffered saline (pH 7.4) containing 1 mM $MgCl_2$ and 0.8 mM $CaCl_2$ (incubation buffer). Cells were then exposed to 200 nM R-MLVP in ice-cold incubation buffer containing bovine serum albumin (1 mg/ml, RIA grade; Sigma) at 4°C for 1 hr. To assess nonspecific binding of R-MLVP an excess of [8-arginine]vasopressin (2 μM) was included in the incubation medium. Cells were then incubated further for various times at 37°C in the presence of medium containing hormone or in fresh buffer only (without added ligand), as noted in the figure legends. Incubations were terminated by removing the medium and rinsing the cells with a 2% solution of paraformaldehyde in incubation buffer. Cells were fixed in the same buffer for 1 hr at room temperature. After coverslips were rinsed with fresh incubation buffer, they were mounted on microscope slides with Airvol (Air Products, Allentown, PA) containing 2% *n*-propyl gallate (17).

The samples were observed with a Nikon FXA microscope (Fryer, Carpertersville, IL) equipped for epifluorescence (excitation, 510–560 nm; barrier filter, 590 nm) and photographed with a ×60/1.4 n.a. Plan apo objective and recorded on Hypertech film (Microfluor, Stony Brook, NY) at an ASA setting of 1600 (8- to 12-sec exposures). Samples were measured for fluorescence intensity by using the absolute photometric value obtained from the FXA photometer. Fluorescence images were also collected with a SIT video camera (Hamamatsu, Middlesex, NJ) for image enhancement using a PC-based Image 1 image processing system (Universal Imaging, Media, PA) and stored on a Panasonic optical disk recorder (model TQ-2028F).

RESULTS

A rhodamine-labeled derivative of vasopressin was used to visualize and quantitate the uptake and intracellular localization of vasopressin by LLC-PK_1 and A10 cells in monolayer. Cells were incubated with R-MLVP for 1 hr at 4°C. This preincubation period allowed binding of the R-MLVP to cell surface vasopressin receptors in the absence of ligand–receptor complex internalization. Upon warming to 37°C, the endocytic pathway becomes active, resulting in the internalization of the ligand–receptor complex, which may be visualized using fluorescent microscopy.

Internalization of R-MLVP and F-MLVP by LLC-PK_1 Cells. When rhodamine excitation–emission of washed and fixed cells was monitored, cell-associated R-MLVP fluorescence was observed that varied in regard to intracellular distribution as a function of time at 37°C (Fig. 1). At the end of the preincubation period, diffuse fluorescence was noted on the cell surface. Early in the 37°C incubation, an array of punctate areas of fluorescence was observed just beneath the basolateral plasma membrane. Upon further incubation, the areas of punctate fluorescence distributed further into the cells, eventually concentrating in the perinuclear region. The specificity of this pattern of punctate fluorescence was indicated by blockage of the formation of the punctate fluorescence in the presence of an excess of [8-arginine]vasopressin. When R-MLVP was maintained in the incubation medium during the 37°C incubation period, punctate areas of fluorescence, which had disappeared initially, were again noted just beneath the basolateral plasma membrane after 50 min of continued exposure to the ligand. This suggests that a new cycle of receptor-mediated endocytosis occurred as a result of cell surface receptor replenishment. When R-MLVP was present in the medium during the 37°C incubation, diffuse fluorescence was apparent within cells along with the punctate areas of fluorescence described above. If cells were exposed to R-MLVP only during the preincubation this pattern of diffuse background fluorescence was not present, resulting in a clearer pattern of punctate fluorescence. If an excess of [8-arginine]vasopressin was included with R-MLVP in the medium at 37°C, punctate areas of fluorescence were not observed; however, diffuse fluorescence was still apparent within cells. The effect of the presence of R-MLVP during the 37°C incubation was also apparent when cellular fluorescence was quantitated. When F-MLVP was used to examine cellular uptake, a pattern similar to that observed with R-MLVP was noted, except that quenching of fluorescein fluorescence was noted in vesicles in the perinuclear region.

Internalization of R-MLVP by A10 Cells. In A10 cells, punctate areas of fluorescence were observed that varied in regard to their intracellular distribution as a function of the time of incubation at 37°C (Fig. 2). The specificity of this pattern of punctate fluorescence was indicated by blockage of the formation of all punctate fluorescence in the presence of an excess of [8-arginine]vasopressin. Fluorescence was diffusely distributed on the surface of A10 cells at the end of the preincubation. After 2 min at 37°C, punctate areas of fluorescence were observed just inside the plasma membrane. These punctate areas of fluorescence distributed in the cell with further incubation at 37°C. The size and intracellular localization of the punctate fluorescence, however, differed from that observed in LLC-PK_1 cells. In contrast to LLC-PK_1 cells, the punctate areas of fluorescence observed in A10 cells were larger and did not localize in the perinuclear region. Furthermore, diffuse cytoplasmic fluorescence was not observed in A10 cells incubated in the presence of R-MLVP and an excess of [8-arginine]vasopressin during the 37°C incubation period. Also, punctate areas of fluorescence were not observed just beneath the basolateral plasma membrane after 50 min of incubation at 37°C in the presence of R-MLVP, suggesting that the vasopressin receptor did not cycle back to the surface in these cells.

DISCUSSION

LLC-PK_1 and A10 cells are established cell lines of renal and vascular origin that contain V_2 and V_1 types of vasopressin receptors, respectively. We employed these cells in culture as model systems to ascertain whether vasopressin is internalized by receptor-mediated endocytosis upon binding with either receptor type.

R-MLVP binding at 4°C occurred diffusely on the surface of both cell types. In LLC-PK_1 cells, which are polar epithelial cells, surface binding was restricted to the basolateral

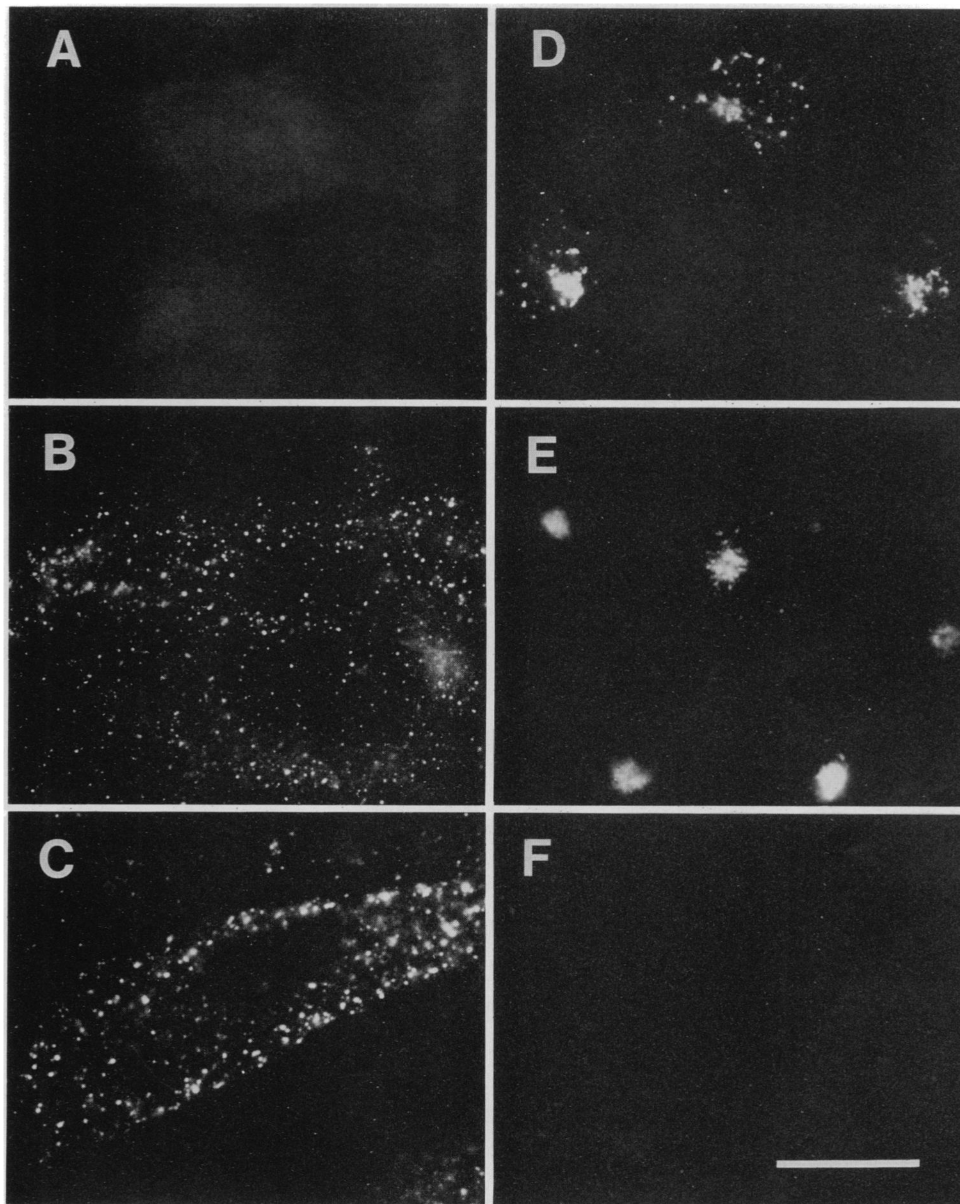

FIG. 1. Localization of R-MLVP in LLC-PK_1 kidney epithelial cells. Cells were incubated at 4°C for 1 hr in 200 nM R-MLVP, washed twice in ice-cold incubation medium without R-MLVP, and then placed at 37°C. Cells were fixed at 0, 4, 8, 20, and 60 min (*A–E*, respectively) following warm-up to 37°C. Cells fixed at the end of the preincubation period (*A*) displayed a diffuse pattern of fluorescence distributed on the cell surface. Within 4 min (*B*) the fluorescence pattern was a punctate localization on the basolateral surface of the cells. By 8 min (*C*) the cells demonstrated an internalization of the punctate distribution. At 20 min (*D*) the punctate pattern had aggregated to the perinuclear region of the cells and by 60 min (*E*) the pattern had concentrated to dense accumulation of spots in the perinuclear region of all five cells in the field (*E*). LLC-PK_1 cells that were treated as above but with the addition of excess unlabeled [8-arginine]vasopressin (1 μM) during all incubations demonstrated competition of binding and internalization of R-MLVP up to 60 min (*F*). (Bar = 10 μm.)

surface. In A10 cells, binding occurred over the entire cell surface. Warming cells to 37°C caused a redistribution of the fluorescent ligand (and presumably its receptor) into intensely fluorescent punctate areas beneath the basolateral membrane in LLC-PK_1 cells and beneath the entire plasma membrane of A10 cells. In both cell types the specificity of this punctate fluorescence was indicated by its elimination in the presence of an excess of [8-arginine]vasopressin. This pattern of punctate fluorescence is consistent with receptor-mediated endocytosis, which has been demonstrated for other peptide hormones (18–21). With continued incubation at 37°C, punctate fluorescence was distributed further within both cell types. However, LLC-PK_1 and A10 cells differed in the size and intracellular location of punctate fluorescence. In LLC-PK_1 cells, punctate fluorescence became concentrated in a single tight aggregate near the perinuclear region. This distribution pattern suggests that the ligand was being distributed to the region of the Golgi apparatus and the lysosome compartment. Whether the ligand was still accompanied by its receptor is not known. Other peptide hormones that exhibit the same endocytic pathway undergo sorting into an endosome/pre-lysosome compartment; the ligand is directed to lysosomes for degradation and the receptor is recycled to the plasma membrane (22–24). When F-MLVP, a fluorescein-labeled vasopressin analog, was used to study vasopressin internalization by LLC-PK_1 cells, initially the same pattern of distribution of fluorescence was observed. By 20 min at 37°C, however, no punctate fluorescence was apparent. This observation indicates that F-MLVP had entered an acidic environment that quenched the fluorescent properties of

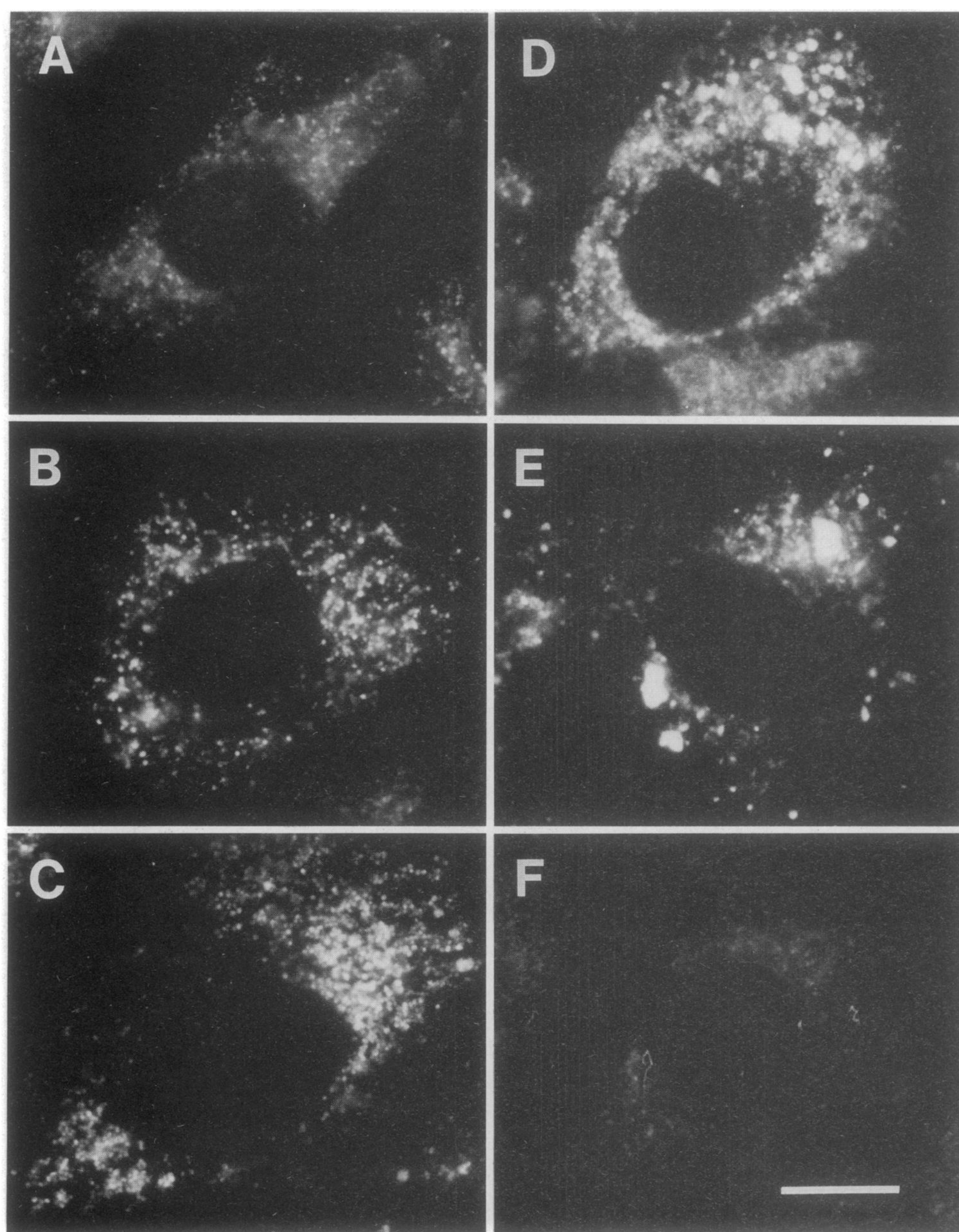

FIG. 2. Localization of R-MLVP in A10 smooth muscle cells. Cells were incubated at 4°C for 1 hr in 200 nM R-MLVP, washed twice in ice-cold incubation medium without R-MLVP, and then placed at 37°C. Cells were fixed at 0, 4, 8, 20, and 60 min (*A–E*, respectively) following warm-up to 37°C. Cells fixed at the end of the preincubation period (*A*) displayed a diffuse pattern of fluorescence distributed on the cell surface. After 4 min at 37°C (*B*) a punctate fluorescent pattern was distributed throughout the cytoplasm. By 8 min (*C*), the punctate spots began to fuse, forming larger vesicles throughout the cytoplasm. The vesicles continued to aggregate and fuse to form larger accumulations in the cytoplasm by 20 min (*D*) and finally form large fluorescent regions by 60 min (*E*). A10 cells that were treated as above but with the addition of excess unlabeled [8-arginine]vasopressin (1 μM) during all incubations demonstrated competition of binding and internalization of R-MLVP up to 60 min (*F*). (Bar = 10 μm.)

fluorescein. Such an acidic environment would cause dissociation of vasopressin from its receptor and allow routing of ligand and receptor to different locations. Fluorescent analogs appear to enter LLC-PK_1 cells by an additional mechanism that does not involve binding to the vasopressin receptor, since it occurs in the presence of an excess of [8-arginine]vasopressin. Furthermore, that this internalization mechanism does not compartmentalize the ligand into discrete punctate areas distinguishes it from receptor-mediated endocytosis. The implications of this alternate internalization mechanism are unclear, as no similar mechanism has been described for the internalization of other peptide hormones. The reappearance of punctate fluorescence beneath the basolateral plasma membrane after an initial concentration of fluorescence in the perinuclear region is consistent with vasopressin receptor recycling in LLC-PK_1 cells. In A10 cells larger punctate fluorescent areas were distributed throughout the cell but did not enter the perinuclear region. The failure of punctate fluorescence to reappear beneath the plasma membrane suggests that internalized vasopressin receptors were not returned to the surface of A10 cells.

Exposure of cells to a hormone results in a diminished response upon subsequent exposure to the hormone. This phenomenon of desensitization occurs with most peptide hormones, including vasopressin (25). In the case of epidermal growth factor and insulin, receptor-mediated endocytosis appears to be an important contributor to desensitization by modulating the number of cell surface receptors (26–28). LLC-PK_1 and A10 cells have been shown to become desen-

sitized in response to vasopressin (29–31). This desensitization is accompanied by a loss of cell surface vasopressin-binding capacity. The results presented here suggest that vasopressin enters these two cell types by receptor-mediated endocytosis. Thus, receptor-mediated endocytosis of vasopressin may be a molecular mechanism by which LLC-PK_1 and A10 cells modulate their responsiveness to vasopressin. The pattern of intracellular distribution of fluorescence observed suggests that the pathway of vasopressin receptor-mediated endocytosis differs in the two cell types. Whether this represents a difference associated with the two vasopressin receptor types or is associated with unique properties of the two cell types is not known.

In summary, we have shown that vasopressin undergoes receptor-mediated endocytosis in cells of vascular and renal origin that contain V_1 and V_2 vasopressin receptor types, respectively. The endocytosis pathway appears to differ in these two cell types and may reflect a difference in the type of vasopressin receptors they contain. Vasopressin receptor-mediated endocytosis in these cells may represent a molecular mechanism for modulating the response of these cells to vasopressin.

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