Dynein and dynactin colocalize with AQP2 water channels in intracellular vesicles from kidney collecting duct

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Ann Taylor, Mark A. Knepper, and Søren Nielsen. Dynein and dynactin colocalize with AQP2 water channels in intracellular vesicles from kidney collecting duct. Am. J. Physiol. 274 (Renal Physiol. 43): F384–F394, 1998.—We investigated whether the motor protein cytoplasmic dynein and dynactin, a protein complex thought to link dynein with vesicles, are present in rat renal collecting ducts and associated with aquaporin-2 (AQP2)-bearing vesicles. Immunoblotting demonstrated cytoplasmic dynein heavy and intermediate chains in kidney, with relative expression levels of inner medulla > outer medulla > cortex. In addition to being present in cytoplasmic fractions, dynein was abundant in membrane fractions enriched for intracellular vesicles. Dynactin was also abundant in membrane fractions enriched for intracellular vesicles. Furthermore, both dynactin and dynein were present in vesicles specifically immunolabeled using anti-AQP2 antibodies. Immunocytochemistry revealed labeling for dynein in the collecting duct principal cells with a pattern consistent with labeling of intracellular vesicles. Moreover, quantitative double immunogold labeling confirmed colocalization of AQP2 and dynein in the same vesicles at the electron microscopic level. Thus the microtubule-associated motor protein dynein and the associated dynactin complex are present in rat renal collecting duct principal cells and are associated with intracellular vesicles, including those bearing AQP2, consistent with the view that dynein and dynactin are involved in vasopressin-regulated trafficking of AQP2-bearing vesicles.

cytosolic dynein; vasopressin; regulated exocytosis; membrane trafficking

THE MAMMALIAN COLLECTING duct represents the final site for the control of water excretion into the urine. Water permeability of the collecting duct is tightly regulated, under the control of the antidiuretic hormone vasopressin, which causes a dramatic increase in collecting duct water permeability, allowing reabsorption of water from the tubular fluid down an osmotic gradient. Indirect evidence obtained in amphibian epithelia led to the “membrane shuttle” hypothesis (37), and it has recently been directly demonstrated that the increased water permeability in the renal collecting duct is brought about by the vasopressin-induced transfer of aquaporin-2 (AQP2) water channels from intracellular vesicles to the apical plasma membrane (15, 21, 38). When vasopressin is removed, water permeability returns to basal levels, reflecting endocytic retrieval of AQP2 water channels (15), which may subsequently be available for reuse (6, 7).

Part of the initial evidence that led to the membrane shuttle hypothesis was the observation that the response to vasopressin was inhibited by drugs that disrupt the cytoskeleton (14, 19, 29, 30). In particular, it was suggested that microtubules might provide a pathway and/or the motive force for the delivery of water channels from an intracellular store to the apical plasma membrane (14, 31). More recent studies in a variety of systems have demonstrated that intracellular trafficking of vesicles can occur along microtubules and that such transport may be driven by microtubule-associated mechanoenzymes (or motors). Such motors can be divided into two groups by the direction in which they move along microtubules, which have an intrinsic polarity. In general, microtubules within cells arise from organizing centers, where their minus (slow-growing) ends are anchored, whereas their plus (fast-growing) ends project away from the organizing centers. Thus minus end-directed (or retrograde) motors, such as cytoplasmic dynein, will transport vesicles toward the organizing center, whereas plus end-directed (or anterograde) motors, such as most of the kinesin family, will drive movement away from the organizing center (25, 35).

Recent studies suggest that microtubules in polarized epithelial cells in culture are organized with microtubules nucleated from multiple organizing centers in the apical part of the cells and project down toward the basolateral surface (13). In addition, there may be a complex mesh of microtubules close to the apical surface (2). If microtubules are organized in this way in collecting duct principal cells, and recent evidence is consistent with this view (31), then a minus end-directed motor such as cytoplasmic dynein might be expected to mediate the transport of AQP2-containing vesicles to the apical plasma membrane upon vasopressin stimulation. Indirect evidence that supports this hypothesis has recently been reported: a relatively specific inhibitor of dynein, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), inhibits the antidiuretic effect of vasopressin in toad urinary bladder, a functional analog of the mammalian collecting duct (10), and the presence of dynein has been demonstrated in bovine and rabbit renal cortex and medulla, as well as in rat kidney (1, 31, 39). It has been shown using in vitro assays that highly purified dynein can cause...
microtubule gliding (26) but is unable to translocate vesicles along microtubules without additional cofactors (24), one of which is the protein complex dynactin (4). The principal component of dynactin, called actin-related protein 1, or Arp1, is a 45-kDa protein thought to be involved in dynactin binding to vesicles, whereas dynactin binding is provided by the 150-kDa components (23, 35). Both of these subunits have been provisionally identified in bovine kidney (1). Among the other constituents of the dynactin complex is a 62-kDa intermediate chain, called p62.

In the present study, we provide evidence, using immunoblotting, immunooisolation of vesicles, immuno- cytochemistry, and immunoelectron microscopy, that dynein and dynactin are present in rat kidney cortex and inner medulla. In the rat kidney and inner medulla, dynein and dynactin are associated with intracellular vesicles, including those containing the vasopressin-regulated water channel AQP2, directly indicating a role of dynein in the vasopressin-regulated trafficking of AQP2 and the consequent alteration of the apical plasma membrane water permeability.

MATERIALS AND METHODS

Antibodies

The monoclonal antibody against cytoplasmic dynein intermediate (70.1) chains has previously been characterized (27). A monoclonal antibody against dynein heavy chain (D1667) was obtained from Sigma Chemical, as was the antibody against ciliary dynein intermediate chain (D6168). The polyclonal antibody against cytoplasmic dynein heavy chain (Dd1) was very kindly provided for this study by Drs. E. A. Vaisberg and J. R. McIntosh and has been previously described (32).

The monoclonal antibodies against the Arp1 (45.A) and p62 (62.B) components of dynactin have been previously described and characterized (22).

For immunolabeling of AQP2 water channels, immune serum or affinity-purified antibody was used, which has been characterized in detail previously (16). This antibody was raised in rabbit against a synthetic peptide corresponding to the COOH-terminal 22 amino acids of AQP2.

Experimental Animals

Studies were performed on male Münich-Wistar rats weighing 250 g (Møllegard Breeding Centre, Elby, Denmark). The rats were maintained on a standard rodent diet (Altromin, Lage, Germany) with free access to water. Rats were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg), and kidneys and brain were removed. Chickens (2.5 kg), obtained from a local supplier, were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg), and kidneys and brain were removed.

Preparation of Membrane Vesicles and Subcellular Fractionation

Crude membrane fractions. Membrane fractions were prepared as previously described (11). Briefly, the kidneys were divided into cortex, outer medulla, and inner medulla. Each section was minced finely and homogenized in 10 ml of dissecting buffer (0.3 M sucrose, 25 mM imidazole, and 1 mM EDTA, pH 7.2, containing the following protease inhibitors: 8.5 µM leupeptin and 1 mM phenylmethylsulfonyl fluoride), with five strokes of a motor-driven Potter-Elvehjem homogenizer, at 1,250 revolutions/min. This homogenate was centrifuged in a Beckman L8M centrifuge at 4,000 g for 15 min at 4°C to remove nuclei, mitochondria, and any remaining large cellular fragments. The pellet was rehomogenized with three strokes, and the centrifugation was repeated to increase yields. The supernatants were pooled and centrifuged at 200,000 g for 1 h. The resultant pellet was resuspended in ~100 µl of dissecting buffer and was assayed for protein concentration using the method of Lowry.

Subcellular fractionation of kidney regions. Membrane vesicle fractions were prepared using a previously described method (12). Briefly, finely minced kidney inner medulla, outer medulla, or cortex was homogenized as described above. The supernatant (after removal of nuclei, mitochondria, etc., see above) was used for the preparation of low-speed and high-speed vesicle fractions by centrifugation of the supernatant at 17,000 g for 30 min and 200,000 g for 1 h, respectively. These represented fractions enriched for plasma membrane (low speed) and intracellular vesicles (high speed). This procedure produced an intracellular vesicle enriched fraction that contains very little plasma membrane. The pellets were resuspended in dissecting buffer and assayed for protein concentration using the method of Lowry.

Preparation of Brain Membrane Vesicles. Crude membranes and membrane fractions of whole rat and chicken brains were prepared as described above.

Immunooisolation of AQP2-Bearing Vesicles

Membrane fractions enriched either for intracellular vesicles (high speed) or plasma membranes (low speed) from kidney inner medulla were prepared as described above. Magnetic beads (Dynal M-280; Dynal, Oslo, Norway), which were precoated with anti-rabbit immunoglobulin G (IgG) antibodies, were coated with affinity-purified anti-AQP2 antibody (L1358, 2 µg/107 beads). The anti-AQP2 antibody-covered beads were incubated with the membrane fractions overnight at 4°C with continuous agitation, in an incubation buffer containing phosphate-buffered saline (PBS), 2 mM EDTA, and 0.1% bovine serum albumin (BSA). After careful washing in three changes of incubation buffer, the beads were mixed with 100 µl of Laemmli sample buffer and were heated to 60°C for 15 min to solubilize proteins associated with the immunoadsorbed vesicles. The beads were then removed magnetically, and the remaining sample buffer was used for immunoblotting to detect dynein or dynactin components. Controls were treated identically except that nonimmune IgG was substituted for the anti-AQP2 antibody.

Preparation of Partially Purified Dynein From Bovine Kidney Inner Medulla

Cytoplasmic dynein was prepared by ATP extraction from Taxol-polymerized microtubules isolated from bovine renal papilla by the method of Vallee (34) and was partially purified by sucrose density gradient centrifugation but was not monoQ purified; thus the complexes contained both dynein and dynactin elements (1). Cytoplasmic dynein partially purified from bovine renal tissue exists as a multimeric complex made up of dynein heavy, intermediate, and light chains, together with associated polypeptides of 150 and 45 kDa (putative components of the dynactin complex). This dynein has been shown to possess EHNA-sensitive adenosine triphosphatase and motor activities (1).
Electrophoresis and Immunoblotting

The membrane samples were solubilized in Laemmli sample buffer containing 2.5% sodium dodecyl sulfate (SDS). Samples were loaded at 25–50 µg/lane onto 12% or 6–16% gradient SDS-polyacrylamide gels and run on a Bio-Rad mini gel system, and proteins were transferred to nitrocellulose paper by electroblotting. The blots were blocked for 1 h with 5% skimmed milk in 80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, and 0.1% Tween 20, pH 7.5 (PBS-T), and then washed with PBS-T. The blots were then incubated overnight at 4°C with antibody in PBS-T with 0.1% BSA at the following dilutions: 70.1 at 1:50, Dd1 at 1:5,000, D1667 at 1:500, 62B at 1:50, and 45.A at 1:1,000. After washing, the blots were incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (P447, 1:3,000; Dako). After final washing, antibody binding was visualized using the enhanced chemiluminescence system (Amersham International). Controls in which the primary antibody was substituted with nonimmune mouse IgG or where the primary or secondary antibody was omitted revealed no labeling.

Immunocytochemistry

Immunocytochemistry was performed as previously described (16). Rat kidneys (from Wistar rats of 250 g allowed free access to food and water) were perfusion fixed by retrograde perfusion through the abdominal aorta with 8% paraformaldehyde or with 2% paraformaldehyde plus 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Tissue blocks prepared from the kidney inner medulla were postfixed in the same fixative for 2 h, infiltrated for 30 min with 2.3 M sucrose containing 2% paraformaldehyde, mounted on holders, and rapidly frozen in liquid nitrogen. Semithin cryosections (0.85 µm) were obtained with a Reichert-Jung cryoultramicrotome, and the sections were placed on gelatin-coated glass slides. After preincubation with PBS containing 1% BSA and 0.05 M glycine, the sections were incubated with the polyclonal antibody (Dd1) with HRP-conjugated goat anti-rabbit secondary antibody (P448, 1:3,000; Dako). After final washing, antibody binding was visualized by use of the horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (P447, 1:500, 62B at 1:50, and 45.A at 1:1,000). The labeling was visualized by goat anti-rabbit Fab fragments (diluted 1:20; Jackson ImmunoResearch Laboratories, West Grove, PA) for 15 min to block free binding sites. Then, the immunolabeling was repeated with affinity-purified anti-AQP2 (0.4 µg IgG/ml), which was then visualized with goat anti-rabbit IgG conjugated to 5-nm colloidal gold particles (diluted 1:50; BioCell Research Laboratories). Grids were finally washed three times with PBS and then three times with 10 mM imidazole (pH 7.4) and were negatively stained with 1% uranyl acetate. In some experiments, 0.3% Triton X-100 was added to the solution containing the primary antibody. Controls were performed identically, except that nonimmune IgG was substituted for the first or second primary antibody. Grids were analyzed in Philips CM100 or Philips EM208 electron microscopes. Electron micrographs were taken and printed at a final magnification of ×85,000.

Quantification of AQP2 and Dynein Double-Immunolabeling in the Intracellular Vesicle Fraction

A total of 3,685 vesicles was analyzed, and the double labeling was quantified in the following four groups as described previously (17): 1) vesicles labeled only for dynein, 2) vesicles labeled only for AQP2, 3) vesicles labeling both for AQP2 and dynein, or 4) vesicles without labeling at all. The number of vesicles within the four groups was determined, the number of gold particles was counted, and the densities were presented as means ± SE. The double-labeling pattern was analyzed statistically by a chi-square test.

RESULTS

Immunoblotting Demonstrates the Presence of Dynein Intermediate and Heavy Chains in Membrane Fractions From Rat Kidney Inner Medulla

The monoclonal antibodies 70.1 and D1667 were raised against chicken brain dynein, and membrane fractions of chicken and rat brain were used as positive controls, together with dynein purified from bovine kidney (Fig. 1). As shown in Fig. 1B, the antibody against the intermediate chain of cytoplasmic dynein (70.1) labeled a single band in a membrane fraction from rat kidney inner medulla, and this band comigrated with the band seen in the positive controls and also in chicken kidney. No labeling was seen in controls in which the primary antibody was replaced with nonimmune IgG at equal concentration. To demonstrate the presence of the dynein heavy chain, two antibodies, one polyclonal (Dd1) and one monoclonal (D1667), were used. Both antibodies labeled a single band in rat kidney and in positive controls (rat brain and purified dynein), and these bands comigrated. Thus immunoblotting demonstrates the presence of intermediate and heavy chain components of the dynein holoenzyme complex in a membrane fraction from rat kidney inner medulla.
Antibodies Recognize Cytoplasmic, Not Ciliary, Dynein

Collecting duct principal cells have a single central cilium. Although it has not been demonstrated that these cilia contain dynein, we carried out immunoblotting analysis to ensure that the antibodies were not recognizing ciliary dynein in our samples. A comparison was made with an antibody against the intermediate chain of ciliary dynein (D6168), between samples from renal inner medulla and tracheal epithelial cells, a richly ciliated epithelium. The results are summarized in Fig. 2. The antibody against ciliary dynein showed no labeling of inner medulla but showed significant labeling of the ciliary epithelial sample. This labeling was found predominantly in the low-speed fraction, consistent with sedimentation of the cilia with the plasma membrane. Antibodies against cytoplasmic dynein showed no labeling of the tracheal sample. Thus the antibodies against cytoplasmic dynein do not cross-react with ciliary dynein, and ciliary dynein is not a significant component in the principal cells.

Presence of Dynactin in Kidney Inner Medulla

Because recent studies have shown that dynactin, a protein complex that associates closely with dynein, is essential for the microtubule-based transport of vesicles in vitro (4, 26), we tested whether dynactin is present in kidney inner medulla. The monoclonal antibody 45A was used to probe immunoblots to test for the presence of Arp1, the principal component of dynactin, in bovine renal dynein preparations and in membrane fractions from rat and chicken kidney and brain. The results are shown in Figs. 1 and 3. The antibody labeled a single band that comigrates in all samples. The relative abundance of Arp1 in the different tissues was similar to that of dynein (also see Fig. 4). Importantly, dynactin was conclusively identified in the partially purified dynein preparation from bovine kidney inner medulla, as shown in Fig. 1, confirming their close association. The monoclonal antibody 62.B, which recognizes the p62 component of dynactin, also labels the membrane fractions (not shown).

Regional Distribution of Dynein and Dynactin in Kidney

Figure 4 summarizes the distribution of dynein and dynactin in different kidney regions. Dynein labeling was seen in membrane preparations from cortex, outer medulla, and inner medulla. Dynin and dynactin showed a broadly parallel distribution, being most abundant (as a fraction of total membrane protein) in the inner medulla, with lower levels in the outer medulla and cortex, although dynactin was relatively more abundant than dynein in the cortex and outer medulla.

Dynein and Dynactin Are Associated With the Intracellular Vesicle Fraction

The membrane preparation protocol used here produces a low-speed fraction, containing mainly plasma membrane vesicles, and a high-speed fraction highly enriched for intracellular vesicles. The membrane fractionation protocol was designed to exclude plasma membranes from the intracellular vesicle-enriched fraction (12), so labeling of the intracellular vesicle fraction is very unlikely to represent contaminating plasma membrane vesicles. Conversely, it cannot be excluded that there are some intracellular vesicles precipitated in the low-speed fraction. As shown in Fig. 4, labeling was strongest in the high-speed fraction, indicating that membrane-associated dynein and dynactin were predominantly associated with intracellular vesicles, rather than with the plasma membrane. This would be expected if dynein is mediating the transport of AQP2.
bearing vesicles toward the plasma membrane, with dynactin providing the link between dynein and the vesicles.

**Dynactin and Dynactin Are Associated With Immunoisolated AQP2-Bearing Vesicles**

To test for a specific association of dynein or dynactin with AQP2-bearing vesicles, vesicles were immunoisolated with antibodies against AQP2 and then were used for immunoblotting. The immunopurification procedure required an overnight incubation at 4°C and repeated washes. As shown in Fig. 5, vesicles immunoisolated with anti-AQP2 antibodies showed very abundant AQP2 labeling, as expected, whereas controls showed no labeling (Fig. 5A). Immunoblotting of such samples demonstrated the presence of dynactin in these vesicles (Fig. 5B), with a predominant labeling of vesicles immunoisolated from intracellular vesicle-enriched membrane fractions (Fig. 5B, middle lanes labeled HS) and only a weak band in fractions isolated from plasma membrane-enriched fractions. Furthermore, immunoblotting of AQP2-immunoisolated vesicles also revealed significant labeling for dynein (Fig. 5B, right). The association of both dynein and dynactin with AQP2-bearing vesicles strongly indicates a role of dynein/dynactin in AQP2 vesicle translocation.

The labeling for dynein in the immunoisolated vesicles was relatively weaker than for dynactin. This may reflect dissociation of the dynein from the vesicles during the in vitro processing required for membrane fractionation and immunoprecipitation. In contrast, dynactin, believed to be a vesicle-bound receptor for dynein, may have a stronger association with vesicles. Consistent with this, homogenates kept overnight at 4°C before preparation of the membrane fractions showed that dynactin was associated primarily with the intracellular vesicle fraction (Fig. 6), whereas dynein (in the same membrane and cytosolic fractions) was found in large amounts in the cytosolic fraction (Fig. 6).

**Immunocytochemistry Confirms the Presence of Dynein in Collecting Duct Principal Cells**

As shown in Fig. 4, immunoblotting demonstrated that dynein was associated predominantly with the intracellular vesicle fraction. To characterize the intracellular distribution further, immunocytochemistry and double immunolabeling of intracellular vesicle fractions were performed. Figure 7 illustrates the findings of immunocytochemical studies of the distribution of dynein in the inner medulla. Principal cells in the collecting ducts labeled for dynein, with a punctate pattern consistent with labeling of intracellular vesicles.

**Fig. 3.** Immunoblot labeled for dynactin actin-related protein 1 (Arp1; A) and subsequently for dynein (A'). A: immunoblot of membrane fractions from chicken kidney (50 µg), chicken brain (25 and 50 µg), and rat kidney inner medulla (25 µg), together with partially purified bovine dynein. Blot has been labeled with a monoclonal antibody (45.A) recognizing Arp1. A': same blot as in A, reprobed with 70.1, the antibody recognizing the dynein intermediate chain. B: control immunoblot, with nonimmune IgG replacing the primary antibody.

**Fig. 4.** Immunoblots showing the distribution of Arp1 and dynein in different kidney regions, together with rat brain as a positive control. Low-speed (plasma membrane) and high-speed (intracellular vesicle) fractions (50 µg/ lane) have been run for each region. A: immunoblot labeled with a monoclonal antibody (45.A) recognizing Arp1. C: similar immunoblot as that in A, labeled with the monoclonal antibody against the intermediate chain of chicken brain dynein (70.1), showing similar distribution pattern. B and D: control immunoblots, using nonimmune mouse IgG in place of the primary antibody.
Thin limbs of Henle and vascular structures also displayed significant dynein labeling.

**Double Immunolabeling Confirms Colocalization of AQP2 and Dynein on the Same Vesicles**

Freshly prepared vesicles from the high-speed fraction (enriched for intracellular vesicles), which had been extensively washed during the preparation protocol, were lightly fixed with 2% paraformaldehyde and 0.1% glutaraldehyde for 10 min and were applied to carbon-coated grids and labeled for dynein, AQP2, or both, as described in MATERIALS AND METHODS. Electron microscopy revealed very low levels of background labeling. Both dynein and AQP2 antibodies labeled small vesicular structures, and, in some cases, vesicles were labeled for both (Fig. 8). To determine whether this association was statistically significant, the number of 5- and 10-nm gold particles associated with 3,685 vesicles was determined in random micrographs from double-labeled grids. The results are presented in Table 1, which demonstrates that dynein labeling correlates highly significantly with AQP2 labeling of the same vesicles.
DISCUSSION

This study demonstrates that cytoplasmic dynein and dynactin are present in the principal cells of rat renal collecting ducts. Dynein is associated with membrane vesicles, and dynactin, believed to be involved in linking dynein to carrier vesicles, is also present in the same membrane fractions. Dynein and dynactin are both associated with vesicles immunoisolated with antibodies against AQP2, and double immunogold labeling of isolated vesicles also confirms a significant colabeling of dynein with AQP2-bearing vesicles. These data are consistent with the involvement of dynein and dynactin in the microtubule-mediated delivery of AQP2-containing vesicles to the apical region of the principal cells, before their insertion into the plasma membrane, in response to vasopressin.

Dynein and Dynactin Are Membrane Associated

The results presented here demonstrate that cytoplasmic dynein is present in both soluble fractions (Fig. 1A) and in membrane fractions (Figs. 1 and 4) of renal inner medulla. Immunoblotting shows that dynein is associated with membrane fractions and is preferentially found in membrane fractions enriched for intracellular vesicles. This is a consistent finding using antibodies against both heavy and intermediate chains of dynein.
Fig. 8. Immunolabeling of intracellular vesicles for dynein and for AQP2. Vesicles from the high-speed fraction (enriched for intracellular vesicles) were labeled for both dynein, visualized with a secondary antibody linked to 10-nm gold particles (arrowheads), and AQP2, visualized with a secondary antibody linked to 5-nm gold particles (small arrows). A: vesicles are labeled for dynein (small arrows) and AQP2 (arrowheads). Magnification: ×85,000. B: similar vesicles as in A at higher magnification (×129,000). C: immunolabeling control in which the antibody against dynein has been replaced with nonimmune IgG, with subsequent labeling for AQP2 as above. Magnification: ×85,000.
Because this fractionation protocol has been optimized to exclude plasma membrane fragments (12), it should consist essentially entirely of intracellular vesicles, together with fragments of cytosolic organelles such as endoplasmic reticulum and Golgi. It is extremely unlikely that cytosolic dynein should be present in the lumen of such vesicles, and dynein in solution will have been very heavily diluted during the extensive wash during the different ultracentrifugation steps. Conversely, the small amount of dynein detected in the low-speed (plasma membrane-enriched) fraction may represent intracellular vesicles sedimented in this pellet and/or soluble dynein trapped within larger membrane vesicles formed from the plasma membrane during homogenization. The finding of a predominant intracellular vesicle association of dynein (and dynactin) is consistent with previous observations in skate hepatocytes and neurons. Immunofluorescence microscopy using an anti-dynein intermediate chain antibody revealed that staining of intact hepatocytes revealed a punctate vesicular pattern. The polarized arrangement of microtubules, the presence of cytoplasmic dynein, and the inhibition of bile salt secretion by nocodazole are consistent with the microtubules playing a fundamental role in the mediation of transcytosis, endocytosis, and bile excretory function in these hepatocytes (5). Furthermore, it has been shown that cytoplasmic dynein is also present as a peripheral membrane protein of purified synaptic vesicles (8). These studies are consistent with the present finding of dynein associated with intracellular vesicles and support current models of microtubule-based organelle translocation. Importantly, immunoblotting also revealed abundant dynactin associated with the membrane fractions enriched for intracellular vesicles (Fig. 4) in a pattern similar to that shown for dynein.

**Dynein is Present in Kidney Collecting Duct Principal Cells**

Immunocytochemistry using HRP labeling and immunofluorescence reveals a distinct labeling of dynein in collecting duct principal cells. Immunolabeling controls were negative, demonstrating that labeling was specific. The immunolabeling of cryosections was relatively sparse, which may suggest that much of the dynein escapes from the tissue during processing, a view that is reinforced by the even weaker labeling seen when formaldehyde alone was used as a fixative. Use of higher concentrations of the polyclonal antibody resulted in significant background labeling, whereas the monoclonal antibodies gave no labeling, probably because the epitope they recognize is not exposed in the folded protein.

The distribution of dynein that we found by immunoblotting, with heavy labeling of membrane fractions from the inner medulla and rather little in the cortex, is consistent with the labeling of dynein from collecting ducts, which make up only a small fraction of the cortex but the majority of the inner medullary tissue. However, it is surprising that only limited immunolabeling was seen in membrane fractions from the cortex, in contrast to the findings of Yoshida et al. (39). It may be speculated that this may reflect the presence of different dynein isoforms in the cortex, which are not recognized by the antibodies used in the present study. Both Yoshida et al. (39) and we (1) have found two dynein heavy-chain bands in samples from kidney cortex, consistent with this view. This may also explain our present observations that dynactin appears relatively more abundant than dynein in the cortex. Furthermore, previous studies showing the presence of dynein in kidney cortex have employed soluble preparations of renal tissue (1, 39), in contrast to the present study in which membrane fractions have been used. Thus it may also be speculated that the lower dynein labeling may reflect a less stable association of dynein with vesicles in the proximal tubule cells than in the collecting duct cells and that the dynein may dissociate from the vesicles during the isolation procedure. Evidence for this is provided by the loss of dynein labeling observed following overnight incubation at 4°C.

### Association of Dynein and Dynactin with AQP2-Bearing Vesicles

The labeling for dynein and dynactin on blots of samples prepared from vesicles immunosolated with antibody against AQP2 reveals that both components are associated with AQP2-bearing vesicles. This is consistent with the hypothesis that the translocation of these vesicles to the apical part of the cell is driven by dynein and may represent a site at which this transport can be controlled, as there is evidence that phosphorylation of dynactin can regulate dynein-mediated transport of vesicles (3). Furthermore, the stable association of dynactin with the vesicle fraction rather than the cytosolic fraction, as well as the abundance in the AQP2-bearing vesicles, in conjunction with the known ability of the dynactin p150 elements to bind dynein (23), provides further support for the view that dynactin acts as a linker between vesicles and the dynein complex.

**Table 1. Quantitation of double immunogold labeling for AQP2 and dynein in vesicles from the intracellular vesicle-enriched (high-speed) fraction**

<table>
<thead>
<tr>
<th>AQP2-labeled vesicles</th>
<th>AQP2-unlabeled vesicles</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td>Dynein Labeled vesicles</td>
<td>159</td>
<td>180</td>
</tr>
<tr>
<td>Dynein Unlabeled vesicles</td>
<td>392</td>
<td>2,954</td>
</tr>
<tr>
<td>Total</td>
<td>551</td>
<td>3,134</td>
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The labeling for dynein and dynactin on blots of samples prepared from vesicles immunosolated with antibody against AQP2 reveals that both components are associated with AQP2-bearing vesicles. This is consistent with the hypothesis that the translocation of these vesicles to the apical part of the cell is driven by dynein and may represent a site at which this transport can be controlled, as there is evidence that phosphorylation of dynactin can regulate dynein-mediated transport of vesicles (3). Furthermore, the stable association of dynactin with the vesicle fraction rather than the cytosolic fraction, as well as the abundance in the AQP2-bearing vesicles, in conjunction with the known ability of the dynactin p150 elements to bind dynein (23), provides further support for the view that dynactin acts as a linker between vesicles and the dynein complex.

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double-labeling studies demonstrated that dynein was associated specifically with intracellular vesicles containing AQP2. This is consistent with the hypothesis that the delivery of AQP2-bearing vesicles to the apical plasma membrane in response to stimulation with vasopressin is driven by cytoplasmic dynein (10, 31), supporting an important role for microtubules in the concerted delivery of AQP2-bearing water channels in response to vasopressin. The observation of a substantial number of vesicles that label for only one of dynein and AQP2 is not unexpected, since dynein probably plays a role in the microtubule-based distribution of other cellular components, whereas AQP2 undergoes endocytosis during periods of diuresis (15). Such endocytotic vesicles would not necessarily be expected to require dynein, given the arrangement of microtubules thought to exist in epithelial cells, where the minus ends are all in the apical part of the cell.

In the unstimulated state, AQP2 labeling is predominantly found in cytoplasmic vesicles spread uniformly throughout the cytoplasm of inner medullary collecting duct principal cells. After vasopressin stimulation, AQP2-bearing vesicles are shuttled to the apical part of the cell, where they are inserted into the apical plasma membrane to increase the water permeability.

Although it is generally accepted that microtubules play a role in the vasopressin-induced increase in amphibian epithelia (31, 33), it has not been clear whether microtubules play a permissive, structural role (33) or whether they are directly involved in the movement of the water channels toward the apical plasma membrane. The observation that microtubule-disruptive agents impaired, but did not abolish, the increase in water permeability caused by vasopressin in amphibian tissue (28, 29, 33) and isolated perfused collecting tubules (19, 20) and suggestions that the main effect was a reduction in the rate of the increase in permeability (33) are consistent with the hypothesis that microtubules provide a pathway along which there is a coordinated delivery of the vesicles to the apical part of the cell. The present study supports the view that dynein and dynactin may play a significant role in this delivery. Preliminary studies indicate a decrease in labeling for dynein and dynactin in intracellular vesicle-enriched membrane fractions after 1-desamino-o-arginine vasopressin treatment (Mariples and Nielsen, unpublished observations) consistent with a role of these components in vasopressin-regulated vesicle trafficking. Recent data have also provided evidence that colchicine-induced depolymerization of microtubules results in the dispersal of AQP2-labeled vesicles throughout the cytoplasm of cortical collecting duct principal cells, in contrast to a polarized localization in the apical and subapical part of the cells in nontreated animals (21).

The final process for insertion of AQP2 into the apical plasma membrane may involve microfilaments (18, 36) and may be regulated by a soluble N-ethylmaleimide-sensitive factor-associated protein (SNAP)/SNAP receptor (SNARE) system analogous to that described in neuronal synapses (17). Indeed both vesicle SNAREs and target membrane SNAREs have been found in kidney collecting duct principal cells (9, 17), and vesicle-associated membrane protein 2 (a vesicle SNARE) has been found to be associated with AQP2-bearing vesicles.

The labeling of the partially purified dynein from bovine kidney inner medulla with the anti-dynactin (Arp1) antibody is expected (Fig. 1), since this dynein has been purified by ATP-dependent microtubule binding and velocity sedimentation, but not by ion exchange chromatography, which is required to separate these two complexes (24). Thus the conclusive demonstration that dynein isolated from kidney inner medulla is associated with dynactin (Fig. 1) supports the view that dynein and dynactin, now shown to be associated with AQP2-bearing vesicles, cooperate in the vasopressin-induced delivery of AQP2-bearing vesicles to the apical plasma membrane.

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Dynein Co-localizes with AQP2 on Vesicles


