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Cellular and subcellular immunolocalization of vasopressin-regulated water channel in rat kidney

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ABSTRACT **Vasopressin (antidiuretic hormone) regulates body water balance by controlling water permeability of the renal collecting ducts. The control mechanisms may involve alterations in the number or unit conductance of water channels in the apical plasma membrane of collecting-duct cells. How this occurs is unknown, but indirect evidence exists for the "shuttle" hypothesis, which states that vasopressin causes exocytic insertion of water channel-laden vesicles from the apical cytosol. To test key aspects of the shuttle hypothesis, we have prepared polyclonal antisera against the recently cloned collecting-duct water channel protein and used the antisera in immunolocalization studies (light and electron microscopic levels) in thin and ultrathin cryosections from rat kidney. Labeling was seen exclusively in collecting-duct principal cells and inner medullary collecting-duct cells. Apical membrane labeling was intense. There was heavy labeling of abundant small subapical vesicles and of membrane structures within multivesicular bodies. In addition, labeling of basolateral plasma membranes in inner medullary collecting ducts was present. Depriving rats of water for 24 or 48 hr markedly increased collecting-duct water-channel protein expression determined by immunoblotting and immunolabeling. These results are compatible with at least two complementary modes of water-channel regulation in collecting-duct cells: (*i*) control of channel distribution between the apical membrane and a reservoir in subapical vesicles (shuttle hypothesis) and (*ii*) regulation of the absolute level of expression of water-channel protein.**

The peptide hormone vasopressin (the antidiuretic hormone) controls body water balance by regulating renal water excretion. Vasopressin does so by controlling the water permeability of the renal collecting ducts, allowing variable reabsorption of water from the duct lumen (urinary space) to the peritubular capillaries. The collecting duct, the terminal part of the renal tubule, is an epithelial structure made up in its various parts by three types of cells: intercalated cells, principal cells, and inner medullary collecting-duct (IMCD) cells. The principal cells and IMCD cells are generally considered as the sites of vasopressin-regulated water transport. The intercalated cells are not generally believed to be a site of regulated water transport, although this view has been disputed (1). The rate-limiting barrier for transcellular water transport across the principal and IMCD cells is thought to be the apical plasma membrane (which faces the urinary compartment) and not the basolateral membrane.

Water transport across cell membranes of water-permeable epithelia occurs via intrinsic membrane proteins that form channels selective for water molecules (2). One such water-channel protein described in mammalian tissues is the channel-forming integral membrane of 28 kDa (CHIP28), which is the major water-channel protein of erythrocytes (3), present in the renal proximal tubule and descending limb of the loop of Henle (4), and found in several other water-transporting tissues (5). Recently, the nucleotide sequence of a cDNA coding for an additional member of this family of proteins [referred to as the aquaporin family (2)] has been reported by Fushimi *et al.* (6). This collecting-duct water-channel protein (WCH-CD) is expressed chiefly, if not exclusively, in the renal collecting duct. On the basis of this localization, WCH-CD has been proposed to be the vasopressin-regulated water channel.

Vasopressin binds to receptors in the basolateral plasma membrane and, acting through cAMP, increases the water permeability of the apical plasma membrane of principal and IMCD cells. The mechanism whereby the water permeability increases is unknown, although it presumably involves an increase in the number of or unit conductance of water channels in the apical membrane. The most widely accepted theory is the "shuttle" hypothesis (7), which proposes that a reservoir of water channels is contained in the membranes of intracellular vesicles and that vasopressin increases the apical plasma membrane water permeability by triggering exocytosis of these vesicles, delivering the vesicles with their water channels to the apical membrane. Studies using freeze–fracture electron microscopy in collecting ducts or vasopressin-responsive amphibian-bladder epithelia have supported this model by showing that discrete patches (termed clusters or aggregates) of intramembrane particles appear in the apical membrane with vasopressin exposure and disappear with vasopressin withdrawal (7–9). These clusters appear to be localized to clathrin-coated pits (8). The intramembrane particles have been proposed to contain the water channels. One item of evidence that is lacking, but is crucial for verification of the shuttle hypothesis, is direct localization of the water-channel protein at a subcellular level. In the present study, we have prepared polyclonal antibodies to the WCH-CD protein and have used these antibodies to carry out immunohisto- and immunocytochemistry to determine the cellular and subcellular localization of the WCH-CD channel.

MATERIALS AND METHODS

Preparation of Antibodies. Two peptides were synthesized by standard automated solid-phase techniques (10) based on the predicted amino acid sequence of WCH-CD (6). These were as follows: peptide 1 (P1), CEVRRRQSVELHSPQSLPRGSKA (amino acids 250–271 of WCH-CD plus an N-terminal cysteine used for conjugation), and peptide 2 (P2), CELHSPQSLPRGSKA (amino acids 258–271 plus an N-terminal cysteine). These peptides were conjugated to keyhole

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Abbreviations: WCH-CD, collecting-duct water-channel protein; IMCD, inner medullary collecting duct.

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limpet hemocyanin or bovine serum albumin by a cysteine sulfhydryl linkage. Three rabbits were immunized with these conjugates at weekly intervals by using combinations of Freund's complete and incomplete adjuvant. Test bleedings were screened by immunoblots or ELISA. Final titers for anti-WCH-CD(P1) were >64,000 in the two rabbits immunized against peptide 1.

The antiserum against peptide 2 was affinity-purified. A purified IgG fraction (protein A-Sepharose; Pharmacia LKB) was further purified by passage over a 5-thio-2-nitrobenzoic acid-thiol-agarose column to which peptide 2 had been attached covalently. The purified anti-WCH-CD(P2) antiserum was eluted at pH 2.5, followed by rapid titration to pH 7.5.

Preparation of Membrane Fractions. Sprague–Dawley rats (175–200 g) were killed by decapitation after ad libitum water intake, water loading, or water withdrawal for 24 hr. Water loading was accomplished by giving 600 mM sucrose as drinking water for 24 hr (urine osmolality < 300 mosm. Both kidneys were removed into isolation solution (250 mM sucrose/10 mM triethanolamine) and dissected into regions as described in *Results*. Tissues from two rats were pooled and homogenized in isolation solution by using a Teflon pestle glass homogenizer. A crude membrane fraction was isolated as described by Turner and Silverman (11).

Electrophoresis and Immunoblotting of Membranes. Membranes were solubilized in SDS, and the quantity of protein was measured spectrophotometrically. SDS/PAGE was done by using Laemmli buffers (12) on 0.1 × 7 × 9 cm minigels of 12% polyacrylamide. Immunoblotting was done essentially as described by Davis and Bennett (13) with enhanced chemiluminescence autoradiography to visualize sites of antigen–antibody reaction.

Immunohistochemistry and Immunocytochemistry. The immunolabeling studies were done as described (4). Sprague–Dawley or Wistar rats were anesthetized with pentobarbital, and the kidneys were fixed by retrograde perfusion through the abdominal aorta with 8% paraformaldehyde/0.15 M sodium cacodylate buffer, pH 7.2. Tissue blocks from cortex, outer and inner stripe of outer medulla, and four levels of the inner medulla were postfixed for 2 hr, infiltrated with 2.3 M sucrose/2% paraformaldehyde for 30 min, mounted on holders, and rapidly frozen in liquid nitrogen. Thin (0.85 μm) and ultrathin (80 nm) cryosections (14) were incubated with anti-WCH-CD(P2) (1:400–1:1200) or anti-WCH-CD(P1) (1:800–1:5000). The labeling was visualized by using horseradish peroxidase-conjugated secondary antibodies for light microscopy and using protein-A–gold (10-nm particles) or a biotinylated secondary antibody followed by avidin–gold (5-nm particles) for EM. For double-labeling, anti-ecto-5′ nucleotidase (1:5000), a marker for intercalated cells (15), was visualized with biotinylated secondary antibody and alkaline phosphatase-conjugated avidin. Controls done at both light and electron microscopic levels revealed no nonspecific labeling. These controls used (*i*) preimmune sera, (*ii*) antiserum absorbed with excess synthetic peptides, and (*iii*) omission primary or secondary antibody.

RESULTS

Fig. 1*A* shows a representative immunoblot of membrane proteins isolated from rat kidney cortex, outer medulla, and inner medulla. At each level, there was a major band at ≈29 kDa, corresponding to the predicted molecular mass of the WCH-CD channel. In addition, there was a broad band at 35–47 kDa, which is probably due to posttranslational modification of the WCH-CD protein—e.g., glycosylation. All three antisera gave identical results. The two bands were absent with antiserum preabsorbed with an excess of peptide or when preimmune serum was used. Anti-WCH-CD bands exhibited increased intensity from cortex to inner medulla.

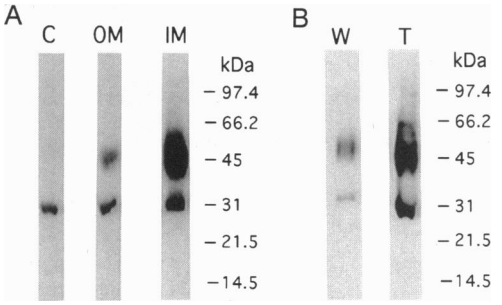

FIG. 1. Immunoblot of proteins from crude membrane fractions. (*A*) Membranes were isolated from cortex (C), outer medulla (OM), and inner medulla (IM) of nonthirsted rat. Ten micrograms of protein was loaded in C and OM lanes, and 1 μg was loaded in IM lane. (*B*) Membranes were isolated from inner medullas of water-loaded (W) and 24-hr thirsted (T) rats. Five micrograms of protein was loaded in each lane. Blots were probed with anti-WCH-CD(P1) antiserum at a 1:5000 dilution.

As illustrated in Fig. 1*B*, WCH-CD expression increased markedly in thirsted (T) relative to water-loaded (W) rats. Similar results were found in six pairs of rats, including experiments in which thirsted rats were compared with random nonthirsted rats.

Figs. 2 (light microscopy) and 3 (electron microscopy) illustrate the key findings of the renal-tissue immunolocalization experiments done in nine rats (six nonthirsted, three thirsted for 48 hr). The anti-WCH-CD(P1) antisera from both rabbits and the anti-WCH-CD(P2) antisera gave identical specific immunolabeling patterns. Anti-WCH-CD antisera preabsorbed with synthetic peptide (Fig. 2 *c-neg* and *d-neg*, and Fig. 3*d*) as well as preimmune sera (data not shown) gave no labeling. Labeling was seen exclusively in collecting ducts (Fig. 2). In cortex and outer stripe of the outer medulla, principal cells were the only cells labeled (Fig. 2 *a* and *b*), and labeling was seen only in the apical region (Fig. 2 *a′* and *b″*). Intercalated cells were not labeled with anti-WCH-CD at any level of the kidney (Figs. 2 *a–d* and 3*b*). This result was confirmed in double-labeling experiments (Fig. 2*b′*).

In the inner stripe of the outer medulla, principal cells exhibited distinct apical labeling, and, in addition, some cells also exhibited basolateral staining (Fig. 2 *c* and *c′*). The basolateral labeling of collecting duct cells was even more intense in the inner medulla (Fig. 2*d*), although the basolateral labeling was less intense than the apical labeling (Fig. 2*e*). In the terminal 30% of the inner medullary collecting duct, labeling appeared weaker and was mainly apical in location (data not shown).

Immunoelectron microscopy of principal and IMCD cells showed that the labeling of the apical region was due, in part, to extensive labeling of the apical plasma membrane (Fig. 3 *a* and *c–h*). Most gold particles were localized to the cytosolic side of the apical membrane, consistent with the proposed cytosolic location of the C-terminal peptides used for immunization. The apical plasma-membrane labeling was not confined to any specific domain (including coated pits) and was particularly prominent over microplicae (Fig. 3 *c*, *e*, and *g*). In addition, in the subapical region of principal and IMCD cells, there was heavy labeling of subapical vesicles (Fig. 3 *a* and *c–f*). Labeling of subapical vesicles was detected throughout the length of the collecting duct in nonthirsted (Fig. 3*g*) as well as thirsted (Fig. 3 *a*, *c*, and *e*) rats. Heavily labeled vesicles were chiefly located in the apical region of cells but were also seen in central and basal parts of the cells (Fig. 3*e*). Labeled coated vesicles were also occasionally observed. Although less abundant, labeling of membranes within multivesicular bodies was also noted (Fig. 3 *e* and *f*). Principal or IMCD cells throughout the outer two-thirds of

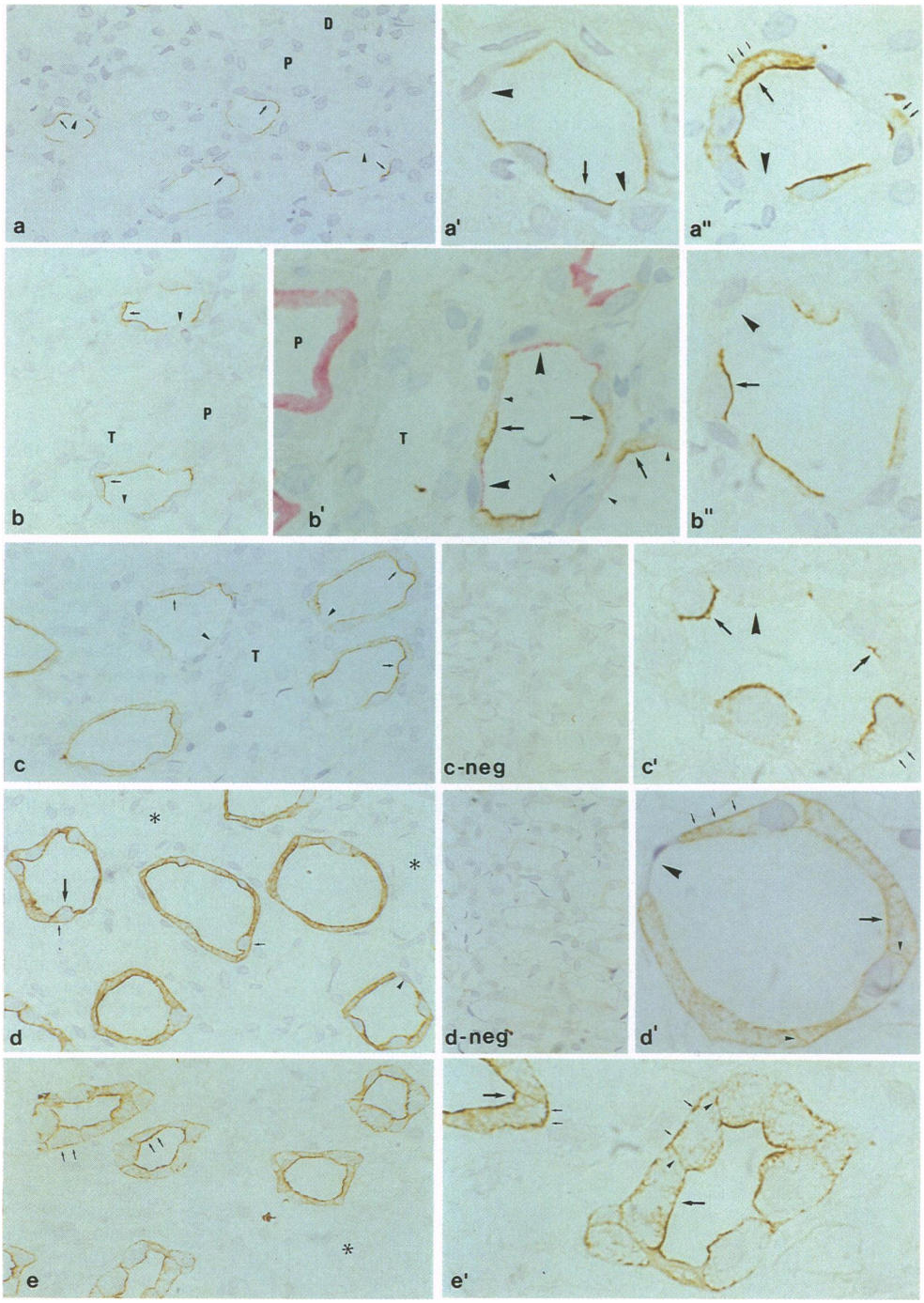

FIG. 2. Immunohistochemical localization of WCH-CD in renal cortex (*a–a″*), outer stripe of outer medulla (*b–b″*), inner stripe of outer medulla (*c–c′*), and different levels in inner medulla (*d–d′*, *e–e′*). All sections except *a″* were from kidneys of nonthirsted rats. (*a*) Outer cortex. Note that collecting-duct principal cells (arrows), but not intercalated cells (arrowheads), were labeled. Proximal tubules (P) and distal convoluted tubule (D) were not labeled. (×300.) (*a′*) Cortex (higher magnification). Labeling of principal cells was confined to apical regions (large arrows). (×770.) (*a″*) Cortex of rat after 48-hr water deprivation. Principal cells exhibit labeling of both apical membrane (large arrow) and basolateral membrane (small arrows). (×770.) (*b*) Outer stripe. Collecting-duct principal cells (arrows), but not intercalated cells (arrowheads), were labeled. Proximal straight tubules (P) and thick ascending limbs (D) were unlabeled. (×300.) (*b′*) Double-labeling for WCH-CD (brown) and intercalated-cell marker ecto-5′-nucleotidase (red) (15) at transition from outer to inner stripe. WCH-CD labeling (arrows) did not overlap ecto-5′-nucleotidase-labeled intercalated cells (large and small arrowheads). Proximal-tubule brush border (P) also exhibited anti-5′-nucleotidase labeling. (×600.) (*b″*) Outer stripe (high magnification). Principal cell apical membranes were labeled by anti-WCH-CD antiserum. (×770.) (*c*) Inner stripe of the outer medulla. Principal cells are labeled predominantly in apical region (arrows). Arrowheads, unlabeled intercalated cells. (×300.) (*c-neg*) Control with peptide-absorbed antiserum. (×220.) (*c′*) Outer medullary collecting duct (high magnification). Note prominent apical labeling of principal cells (large arrows) and moderate labeling of basolateral membranes of some principal cells. (×770.) (*d*) Outer 25% of inner medulla. Principal cells of collecting ducts exhibit intense apical (large arrows) and basolateral (small arrows) labeling. Intercalated cells (arrowhead), vascular structures, and thin limbs (asterisks) were unlabeled. (×300.) (*d-neg*) Control with peptide-absorbed antiserum. (×220.) (*d′*) Collecting duct from base of inner medulla (high magnification). Principal cells exhibit labeling of apical (large arrow), basal (small arrows), and lateral (small arrowheads) regions. (×770.) (*e*) Middle part of inner medulla. IMCD cells exhibit both apical and basolateral labeling (arrows). Thin limbs (asterisk) were unlabeled. (*e′*) IMCD, midinner medulla. IMCD cells exhibited strong apical labeling (large arrows) and less intense labeling of basal (small arrows) and lateral (arrowheads) region. (×770.)

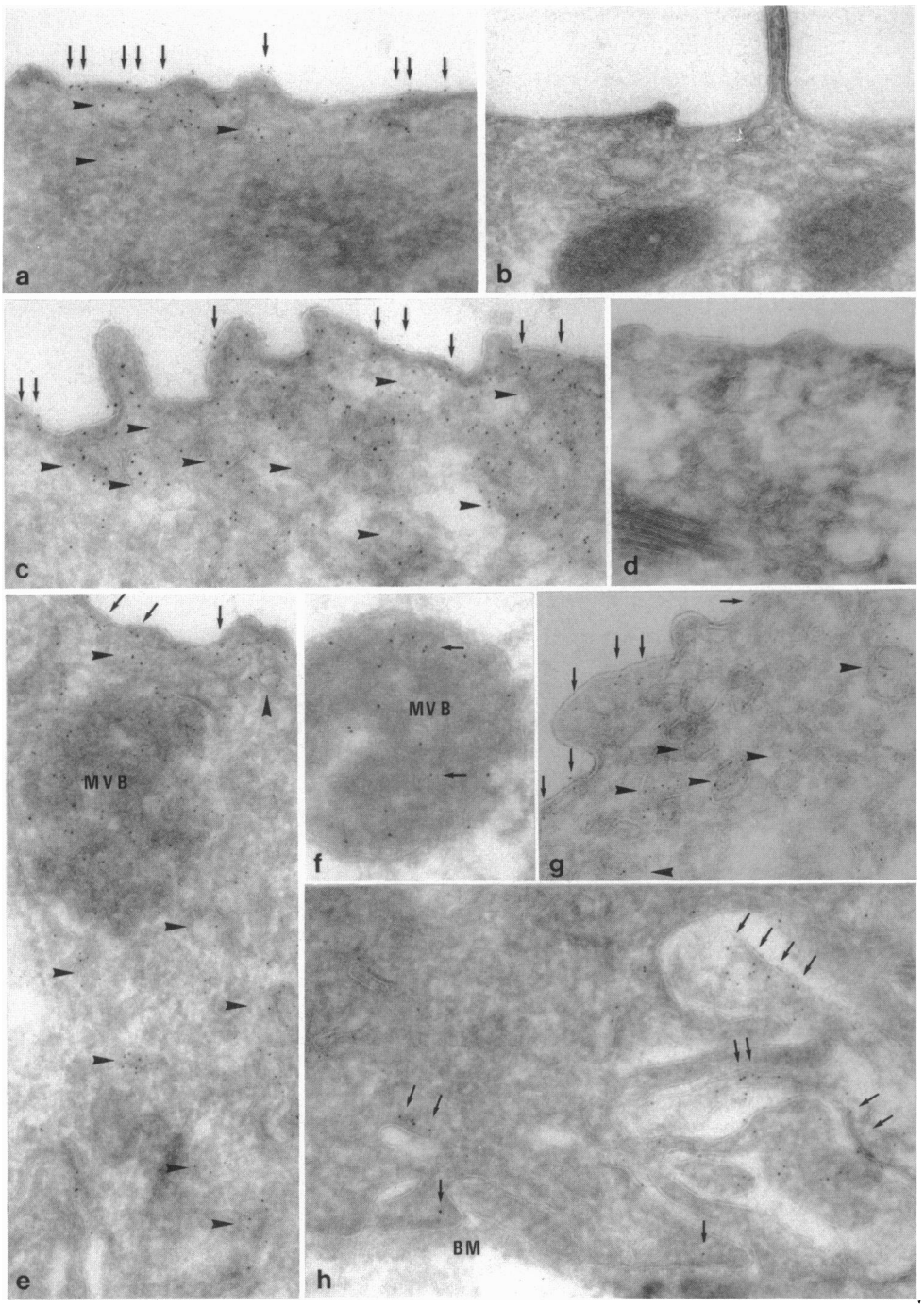

FIG. 3. Immunocytochemical localization of WCH-CD in outer (*a–b*) and inner (*c–h*) medullary collecting-duct cells. Ultrathin cryosections were obtained from thirsted rats (*a–e* and *h*) and nonthirsted rats (*f–g*). (*a*) Principal cell of outer medullary collecting duct. Labeling was confined to apical plasma membrane (arrows) and subapical vesicles (arrowheads). (*b*) Intercalated cell of outer medullary collecting duct; note absence of labeling. (*c*) IMCD cell, showing intense labeling of apical plasma membrane (arrows) and of cross- and tangentially sectioned subapical vesicles (arrowheads). (*d*) IMCD cell; control with peptide-absorbed antiserum. (*e*) IMCD cell from midinner medulla, exhibiting labeling of apical plasma membrane (arrows) and of vesicles positioned subapically and deeper in cytoplasm (arrowhead). Also, a multivesicular body (MVB) is labeled. (*f*) IMCD cell, multivesicular body (MVB). Labeling (protein-A–gold) is associated with vesicular structures (arrows). (*g*) IMCD cell (nonthirsted rat) exhibiting labeling of apical plasma membrane (arrows) and subapical vesicles (arrowheads). (*h*) IMCD cell. Intense anti-WCH-CD labeling of basolateral membranes including basal infoldings (arrows). BM, basement membrane. (*a–d*, *g–f*, ×53,000; *e* and *f*, ×42,000.)

the inner medulla exhibited intense labeling of the basolateral plasma membrane, especially the lateral infoldings and interdigitations (Fig. 3*h*).

Consistent with the increase in WCH-CD expression seen in immunoblots (Fig. 1*B*), depriving rats of water for 48 hr appeared to markedly increase labeling intensity by immunohistochemistry [compare Fig. 2*a'* (nonthirsted) with 2*a''* (thirsted), in which labeling techniques were identical]. Furthermore, at an ultrastructural level, increased labeling of plasma membranes and subapical vesicles was noted in collecting-duct cells of water-deprived rats [compare Fig. 3 *c* and *e* (thirsted rats) with Fig. 3*g* (nonthirsted rat)].

DISCUSSION

Immunohistochemistry and immunocytochemistry with antisera raised against the C-terminal region of the rat WCH-CD revealed exclusive labeling of collecting-duct cells in rat kidney. The pattern of labeling was identical for all antisera used. Immunoblots demonstrated that the antiserum recognizes a 29-kDa band, consistent with the predicted molecular mass of unmodified WCH-CD, plus a larger band consistent with the presence of a glycosylated form. Peptide-absorption controls in both immunoblots and immunolocalization studies showed the specificity of the antisera. Labeling was clearly demonstrable throughout the length of the collecting-duct system and was selective for the cell types generally believed responsible for vasopressin-mediated regulation of collecting-duct water absorption—namely, the principal cells and IMCD cells. These results, therefore, are consistent with reverse transcription PCR experiments of Fushimi *et al.* (6) showing that WCH-CD mRNA is confined to collecting-duct segments, as well as with preliminary immunofluorescence experiments (using a similarly derived antiserum on cryostat

sections) showing labeling of collecting-duct cells. Localization of the WCH-CD channel exclusively to collecting ducts supports the suggestion of Fushimi *et al.* (6) that this water channel is the vasopressin-regulated water channel because isolated perfused tubule studies have demonstrated that vasopressin regulates water permeability only in collecting ducts and does not regulate water permeability in the various nephron segments or in connecting tubules (for review, see ref. 16). No labeling of intercalated cells was seen.

Electron microscopic examination of principal and IMCD cells showed prominent labeling of the apical plasma membrane, which is the rate-limiting barrier for transepithelial water transport and is the site at which vasopressin regulates membrane water permeability (1, 17). The labeling was distributed throughout the apical membrane (including microplicae and invaginations) and was not confined to coated pits. This distribution appears contrary to the view that vasopressin-regulated water channels may reside exclusively in restricted domains identified in freeze–fracture experiments as intramembrane particle clusters (7–9), although this distribution does not discount the proposed role of these clusters in the delivery of water channels to the apical membrane. Importantly, our results show that WCH-CD channels are present in other structures besides the apical plasma membrane—namely, small subapical vesicles, multivesicular bodies, and (in portions of the medulla) the basolateral membrane. Thus, there exists a large intracellular reservoir of vesicle-associated water-channel protein. This water-channel reservoir appears strategically located, so that it is available for recruitment into the apical membrane by vasopressin-stimulated exocytosis, the central tenet of the shuttle hypothesis (7). In addition, the localization of the WCH-CD to multivesicular bodies supports the view that these structures may be involved in processing water channels retrieved from the apical membrane by endocytosis after vasopressin withdrawal. The physical appearance and site of subapical vesicles and multivesicular bodies labeled by the anti-WCH-CD antiserum are in full accord with structures that incorporate endocytic markers from the lumen after vasopressin withdrawal in isolated perfused IMCD segments (18), lending further support to the view that these structures represent an endo/exocytic compartment.

Basolateral labeling of medullary collecting-duct cells was a consistent finding with each of our anti-WCH-CD antisera. We do not know whether the basolateral labeling was due to the presence of WCH-CD itself or possibly of an as-yet-unidentified water channel that may share structural similarities with WCH-CD. Indeed, recent expression studies in oocytes indicate that at least one additional water channel may be expressed in the renal medulla aside from the 28-kDa channel-forming integral-membrane protein (CHIP28) (expressed in thin descending limbs) and WCH-CD (19).

Water restriction of rats markedly enhanced WCH-CD expression, as assessed by immunoblotting (Fig. 1*B*). The immunolocalization studies (both light and electron microscopy) showed that the increased expression was associated with an increase in WCH-CD labeling in principal and IMCD cells, including an apparent increase in basolateral and vesicular expression. These results therefore suggest that, in addition to recruitment of water channels into the apical membrane from water-channel-laden vesicles, water-channel regulation is associated with an increase in water-channel expression in collecting-duct cells. The present findings are fully compatible with our earlier results showing that water restriction of rats increases the water permeability of their IMCDs when perfused *in vitro*, independent of the acute effect of vasopressin (20).

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