Identification of Rab3-, Rab5a- and synaptobrevin II-like proteins in a preparation of rat kidney vesicles containing the vasopressin-regulated water channel

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Abstract According to the 'shuttle hypothesis', vasopressin increases the water permeability of renal epithelial cells by exocytotic fusion of vesicles containing the water channel AQP-CD with the apical plasma membrane, whereas withdrawal of vasopressin results in endocytotic uptake of AQP-CD. The proteins involved in the redistribution of AQP-CD have not been identified. With a panel of monoclonal antibodies, we detected Rab3-, Rab5a- and synaptobrevin II-like proteins in a kidney preparation enriched in AQP-CD-containing vesicles. The synaptobrevin II-like protein is not identical with the ubiquitous cellubrevin. Rab3- and synaptobrevin II- but not Rab5a-like proteins were co-enriched with AQP-CD. The data suggest that the proteins involved in hormonal regulation of water permeability in kidney epithelial cells are identical or similar to those involved in regulated exocytosis in secretory cells.

Key words: Antidiuresis; Water channel; AQP-CD; Exocytosis; Small GTP-binding protein; Synaptobrevin

1. Introduction

The peptide hormone vasopressin, also referred to as antidiuretic hormone, is the key regulator of water homeostasis in mammals. The main site of vasopressin action is the renal collecting duct. Vasopressin binds to V2 receptors [1] expressed on the basolateral surface of epithelial (principal) cells and, acting via the adenylyl cyclase cascade, increases the water permeability of the apical plasma membrane. This permits the reabsorption of water from the lumen of the collecting duct.

Water transport across epithelia is mediated by intrinsic membrane proteins (aquaporins, AQPs), which form selective channels for water molecules (for review see [2]). The first AQP to be cloned was AQP-CHIP, a highly abundant protein in erythrocyte membranes [3]. In the kidney, AQP-CHIP, located in epithelial cells of the proximal tubule and of the descending loop of Henle, seems to be required for constitutive water transport [2]. AQP-CD (formerly called WCH-CD) is exclusively located in the renal collecting duct [4]. The cellular distribution of AQP-CD and the finding that inactivating mutations are associated with an atypical form of congenital nephrogenic diabetes insipidus [5] provide strong evidence that AQP-CD is the vasopressin-regulated AQP. AQP-CD is the only known water-channel exhibiting fast hormonal regulation.

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Abbreviations: AQP-CD, aquaporin-collecting duct; GTPS, guanosine 5′-[γ-thio]triphosphate.

The molecular mechanisms of the vasopressin-induced increase in water permeability of principal cells are poorly understood. According to the 'shuttle hypothesis' (for reviews see [6,7]), vasopressin increases water permeability by exocytotic insertion of water channels in the apical membrane. This hypothesis is strongly supported by the recent finding that AQP-CD is associated with the apical plasma membrane and with subapical and perinuclear vesicles of principal cells [8]. In agreement with previous reports, Harris et al. [9] have shown that specific endosomal vesicles from collecting duct epithelia contain AQP-CD. This observation is consistent with a redistribution of apical water channels into specialized vesicles upon removal of vasopressin stimulation. Together, the data support the idea that AQP-CD-containing vesicles undergo hormone-controlled exo-/endocytosis, resembling regulated exo-/endocytosis in nerve terminals.

In recent years considerable progress has been made in elucidating the molecular mechanisms underlying the assembly, docking and fusion of vesicles serving intracellular transport and exocytosis. A basic docking/fusion machinery appears to be operating in all cells [10]. Additional components seem to be required to determine the nature of the docking/fusion process (e.g. exocytosis versus endocytosis, constitutive exocytosis versus regulated exocytosis). For example, proteins of the Rab3 and Rab5 families are specifically linked to regulated exocytosis and to endocytosis, respectively [11].

To gain insight into the vasopressin-regulated increase in water permeability, we looked for proteins involved in vesicular transport in a preparation from rat kidney enriched in AQP-CD containing vesicles. The present data show that this preparation contains proteins detected by an antibody which recognizes Rab3a, b and c and by antibodies specific for Rab5a and synaptobrevin II. The identification of immunoreactive proteins in the vesicle preparation strongly indicates that vasopressin regulates water permeability by exo-/endocytotic mechanisms similar to those in secretory cells.

2. Materials and methods

2.1. Materials

Phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, aprotinin and benzamidine were from Sigma (München, FRG), recombinant *N*-glycosidase F from Boehringer (Mannheim, FRG), Percoll from Pharmacia (Freiburg, FRG), nitrocellulose membranes from Schleicher and Schuell (Dassel, FRG), ^{125}I-labeled donkey anti-rabbit IgG (185–740 kBq/μg) and ECL (enhanced chemiluminescence) Western-blotting detection system from Amersham Buchler (Braunschweig, FRG), and ^{125}I-labeled goat anti-mouse IgG (74–370 kBq/μg) from DuPont (Bad Homburg, FRG).

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2.2. Antisera

For the generation of polyclonal antisera against AQP-CD a synthetic peptide (VELHSPQSLPRGSKA) corresponding to the 15 C-terminal amino acids of AQP-CD with an additional N-terminal tyrosine residue [4] was coupled to keyhole limpet hemocyanine and used for the immunization of rabbits.

The following antibodies, kindly provided by Reinhard Jahn (New Haven, CT), were employed: antibody Cl 42.1 recognizes Rab3a, b and c; it is not known whether the antibody also detects Rab3d ([12] and Reinhard Jahn, personal communication); antibody Cl 621.2 recognizes Rab5a only ([13] and Reinhard Jahn, personal communication); antibody Cl 69.1 recognizes synaptobrevin II [14], antibody Cl 10.1 recognizes cellubrevin and synaptobrevins I and II [15]; antibody Cl 41.1 recognizes synaptotagmin I [16]; antibody Cl 43.1 recognizes synaptophysin [17].

2.3. Purification of AQP-CD-containing vesicles from rat renal inner medulla and papilla

Isolation of AQP-CD-containing vesicles was performed according to Harris et al. [9] with minor modifications. All steps were performed at 4°C, and all buffers were supplemented with a mixture of protease inhibitors (0.5 mM PMSF, 3.2 μg/ml soybean trypsin inhibitor, 2.8 μg/ml aprotinin, 1 mM benzamidine). The inner medullae and papillae from 20 rat kidneys were minced and homogenized by 10–15 strokes in buffer A (300 mM mannitol, 12 mM HEPES, pH 7.6) using a glass/teflon homogenizer. The homogenate was centrifuged at 2,500 × *g* for 20 min. The supernatant was centrifuged at 20,000 × *g* for 20 min. The resulting supernatant and the upper layer of the pellet were combined, and centrifuged at 48,000 × *g* for 30 min. The resulting pellet was resuspended in buffer A, homogenized with 30 strokes, and Percoll was added to 18% (wt/wt). The suspension was centrifuged at 48,000 × *g* for 30 min; the bottom one-third was collected and diluted with an 8-fold volume of buffer B (300 mM mannitol, 100 mM KCl, 5 mM $MgSO_4$, 5 mM HEPES, pH 7.6). After incubation for 15 min, the suspension was centrifuged at 48,000 × *g* for 30 min. The pellet was resuspended in buffer B and recentrifuged at 5,000 × *g* for 15 min. The supernatant was discarded and the fluffy upper part of the pellet, containing renal papillary and medullary vesicles, was carefully separated from the brown bottom pellet and resuspended in buffer B. The yield from 20 kidneys was about 1 mg of protein. The preparation was stored at −80°C.

The activity of Na^+/K^+-ATPase, employed as marker enzyme for the basolateral plasma membrane, was determined by a coupled spectrophotometric assay in the absence or presence of ouabain as described [18].

2.4. Preparation of membranes

All procedures were performed at 4°C. Membranes from kidney medulla and papilla were prepared according to Kinne-Saffran and Kinne [19] with minor modifications. Briefly, the dissected tissue was homogenized with a glass/teflon homogenizer in a buffer consisting of 250 mM sucrose, 10 mM triethanolamine/HCl (pH 7.6) and centrifuged at 700 × *g* for 10 min. The supernatant was centrifuged at 10,000 × *g* for 10 min. The supernatant and the homogenized pellet were recombined and centrifuged at 100,000 × *g* for 60 min. The pellet was resuspended in the buffer described above.

N-glycosidase F digestion was performed according to the manufacter's protocol. Briefly, the membrane pellet (see above) was resuspended in a buffer consisting of 50 mM EDTA, 10 mM NaN_3, 1% SDS and 20 mM sodium phosphate (pH 7.2). Aliquots (50 μg of protein in 10 μl) were boiled for 2 min. Ninety μl of the same buffer supplemented with 0.5% Nonidet P-40 but without SDS were added and the sample was again boiled for 2 min. *N*-glycosidase F (1 unit) was added, and the sample was incubated for 20 h at 37°C. Samples were precipitated with acetone before analysis by SDS PAGE/immunoblotting.

Rat brain was homogenized by 10 strokes with a glass/teflon homogenizer in a buffer consisting of 1 mM EDTA, 0.25 mM DTT and 10 mM HEPES (pH 7.4) and centrifuged at 10,000 × *g* for 15 min. The supernatant was centrifuged for 90 min at 120,000 × *g*. The resulting pellet was resuspended in the same buffer and stored at −80°C.

L cells (mouse fibroblasts) were cultured in Dulbecco's modified Eagle medium containing 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). For preparation of a crude membrane fraction, cells were rinsed twice with phosphate-buffered saline consisting of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1 mM KH_2PO_4 (pH 7.4), scraped off the dishes and centrifuged at 200 × *g* for 5 min. The pellet was resuspended in a buffer consisting of 27% (w/v) sucrose, 1 mM EDTA, 20 mM HEPES (pH 7.8) and homogenized with 10 strokes in a glass/teflon homogenizer. The pellet obtained by centrifugation at 20,000 × *g* for 10 min was resuspended in phosphate-buffered saline. The preparation was stored at −80°C.

2.5. Immunoblotting

Immunoblotting was performed as described [20]. Briefly, proteins were resolved by SDS PAGE (30–60 μg/lane, 12 or 15% polyacrylamide) and transferred onto nitrocellulose filters. The filters were blocked by incubation with Tris-buffered saline (137 mM NaCl, 0.01% sodium azide, 10 mM Tris-HCl, pH 7.5) supplemented with 1% (v/v) gelatin and 1% (w/v) ovalbumin, and antisera were added for 2 to 12 h. ^{125}I-labeled anti-mouse IgG or ^{125}I-labeled anti-rabbit IgG were used as second antibodies. Filters were autoradiographed for 1–3 days. Detection of proteins with antibody Cl 10.1 was performed with the ECL Western-blotting system according to the manufacterer's protocol.

2.6. Protein determination

Protein was measured according to Lowry et al. [21].

3. Results

A polyclonal antibody was raised against the C-terminal peptide sequence of the rat water channel AQP-CD [4]; this sequence does not occur in any other AQP. The properties of the antibody are very similar to those of previously described antibodies raised against the same epitope (e.g. [8,9]). In membranes from rat kidney inner medulla and papilla, the antibody but not the preimmune-serum labeled a broad band at 35 to 40 kDa, a narrow band at 29 kDa and material above 94 kDa (most likely representing large aggregates of AQP-CD) (Fig. 1A). Labeling was abolished by preincubation of the antiserum with the synthetic peptide, indicating the presence of the antigen in all immunoreactive bands. The assumption that the high-molecular-weight material represents aggregates of AQP-CD is further supported by the finding that AQP-CD has a marked tendency to aggregate even in the presence of SDS (unpublished). Fig. 1B shows that staining of the 35–40 kDa band is significantly reduced by treatment with the enzyme *N*-glycosidase F. This finding indicates that the 35–40 kDa band and the 29 kDa band represent the glycosylated and nonglycosylated forms of AQP-CD, respectively. Also consistent with previous reports [8,9], AQP-CD was found to be more abundant in the inner medulla/papilla than in the cortex (Fig. 1C). The antibody failed to detect proteins in either human erythrocyte membranes (a rich source for AQP-CHIP) or cell lines (CHO, L) expressing functional water channels (not shown). The data indicate that the antibody specifically recognizes AQP-CD.

For further studies, AQP-CD-containing vesicles were purified from rat kidney medulla/papilla according to a recently published method [9]. To check for contamination with basolateral membranes, Na^+/K^+-ATPase activity was determined in both the starting material (homogenate) and the vesicle preparation; three independent preparations were tested. Whereas activity was present in the homogenate (0.19 ± 0.04 U/mg protein; mean ± S.D.), no activity was detectable in the vesicle preparation. This finding is consistent with the absence of basolateral membranes in the vesicle preparation and indicates that the vesicles are not of basolateral origin. Fig. 2A shows that AQP-CD is several-fold enriched in the vesicle preparation

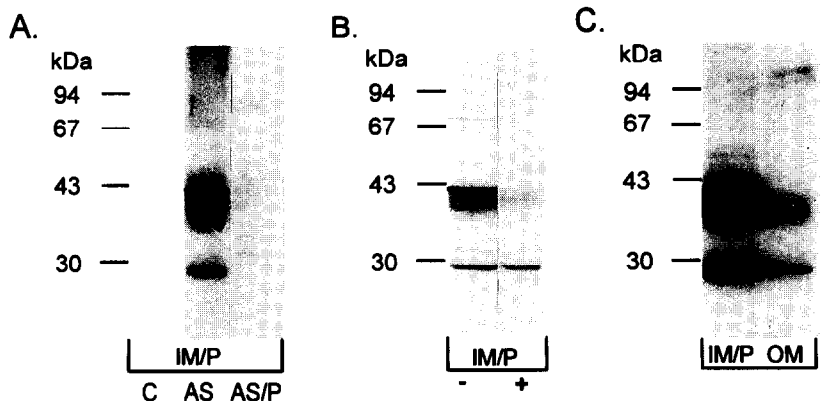

Fig. 1. Characterization of an antibody raised against a synthetic peptide specific for AQP-CD. Various AQP-CD-containing preparations (60 μg of protein per lane) were analyzed by SDS PAGE/immunoblotting. Preimmune serum or anti-AQP-CD antiserum was employed at a 1 : 1,000 dilution. Filter-bound antibody was labeled with ^{125}I anti rabbit IgG antibody. Shown are autoradiograms of filters. (A) Filters from rat inner medulla/papilla (IM/P) were incubated with preimmune-serum (C), anti-AQP-CD antiserum (AS), or anti-AQP-CD antiserum preincubated with the peptide immunogen (35 μg/ml; AS + P). (B) Prior to SDS PAGE/immunoblotting with anti-AQP-CD antiserum, membrane aliquots from IM/P were incubated without (−) or with *N*-glycosidase F (+). (C) Membrane aliquots from IM/P or outer medulla (OM) were analyzed by SDS PAGE/immunoblotting with anti-AQP-CD antiserum.

compared to the homogenate. A panel of monoclonal antibodies (see section 2) was employed to search for proteins known to be involved in vesicular transport. Fig. 2B shows that these antibodies recognize their antigens Rab3 (27 kDa), Rab5a (27 kDa), synaptobrevin II (18 kDa), synaptotagmin I (65 kDa) and synaptophysin (38 kDa) in rat brain membranes. In the vesicle preparation from rat kidney, proteins were detected by Rab3, Rab5a and synaptobrevin II antibodies but not by the synaptotagmin I and synaptophysin antibodies. The mobilities of the immunoreactive proteins in the brain and kidney preparations were indistinguishable, suggesting that the proteins are identical or at least very similar. The antibody against synaptobrevin II does not recognize the ubiquitous synaptobrevin homologue, cellubrevin [22]. Hence the immunoreactive protein in the kidney preparation is not cellubrevin.

Together with AQP-CD, the Rab3- and synaptobrevin II-like proteins were enriched during the course of vesicle purification (Fig. 3). These findings are consistent with a localization of Rab3- and synaptobrevin II-like proteins on AQP-CD-containing vesicles. In contrast, the Rab5a-like protein was not enriched. Among the various antibodies, only the Rab5a antibody detected a protein in L cells, reflecting the occurrence of the protein in both secretory and non-secretory cells [23].

By binding of [^{35}S]GTPγS to proteins immobilized onto nitrocellulose, proteins of about 27 kDa, most likely corresponding to members of the Rab family, and a protein of 20 kDa were

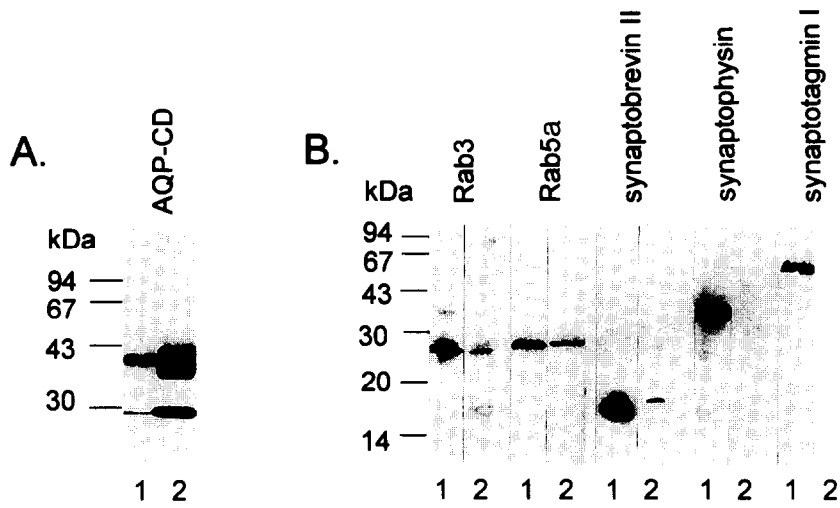

Fig. 2. Detection of Rab3-, Rab5a- and synaptobrevin II-like proteins in AQP-CD-containing vesicles. Proteins were analyzed by SDS PAGE/immunoblotting with the indicated antibodies. Filter-bound antibodies were labeled with ^{125}I anti rabbit or anti mouse IgG antibody. Shown are autoradiograms of filters. (A) Equal amounts of protein (60 μg per lane) from homogenate (lane 1) and AQP-CD-containing vesicles (lane 2) prepared from rat kidney medulla/papilla were analyzed by SDS PAGE/immunoblotting with anti-AQP-CD antiserum. (B) Rat brain membranes (lanes 1, 30 μg of protein) and AQP-CD-containing vesicles from kidney medulla/papilla (lanes 2, 60 μg of protein) were analyzed by SDS PAGE/immunoblotting with monoclonal antibodies specific for Rab3 (Cl 42.1), Rab5a (Cl 621.2), synaptobrevin II (Cl 69.1), synaptophysin (Cl 43.1) and synaptotagmin I (Cl 41.1).

prominently labeled in the vesicle preparation (not shown). The data suggest that AQP-CD-containing vesicles possess other small GTP-binding proteins in addition to Rab proteins.

We also employed an antibody (Cl 10.1), which recognizes synaptobrevins I and II and cellubrevin (see section 2). Short exposure of filters (Fig. 4A) revealed that this antibody recognizes two bands in brain membranes. The upper band comigrated with the protein recognized by the antibody specific for synaptobrevin II (not shown). Thus the upper band recognized by antibody Cl 10.1 is tentatively identified as synaptobrevin II, whereas the lower band may represent synaptobrevin I and cellubrevin, the latter being of non-neuronal origin (glia, vessels). Resolution of the two bands is lost after the long exposure required for detection of proteins in kidney and L cell preparations (Fig. 4B). In starting material from kidney inner medulla/papilla, a protein comigrating with the lower band visible in Fig. 4A is stained by the antibody. In the preparation enriched in AQP-CD-containing vesicles an additional band comigrating with the upper band visible in Fig. 4A is detected. This band most likely represents the synaptobrevin II-like protein. In L cells, a band comigrating with the lower band visible on Fig. 4A is detected; this band may represent cellubrevin. In this cell line the antibody recognizes an additional protein of 12 kDa; the nature of this protein is unknown. The data are consistent with the view that the synaptobrevin II-like protein but not cellubrevin is enriched during the course of vesicle preparation. They provide additional independent evidence for the notion (see above) that the synaptobrevin II-like protein is not identical with cellubrevin.

4. Discussion

In this report we show that a preparation from rat kidney medulla/papilla enriched in AQP-CD-containing vesicles also contains a protein of the Rab3 family and a Rab5a- and a synaptobrevin II-like protein. Previous studies revealed the presence of mRNA for Rab5 but failed to detect mRNA for Rab3 and synaptobrevins in total kidney mRNA ([23] and refs. cited therein, [24]). In contrast to Rab3 proteins expressed in cells exhibiting regulated exocytosis, Rab5 proteins are found in cells both with and without regulated exocytosis [11]. In agreement with Zahraoui et al. [23], a Rab5-like protein was also detected in L cells. Besides its presence in neuronal cells, synaptobrevin II has recently been detected in rat skeletal muscle and in L6 myotubes, and synaptobrevin-like molecules are present in adipocytes and pancreatic acinar cells ([25] and refs. cited therein). The apparent absence of synaptotagmin I and synaptophysin from the vesicle preparation precludes contamination with material from peripheral nerves as a source of Rab3- and synaptobrevin II-like proteins.

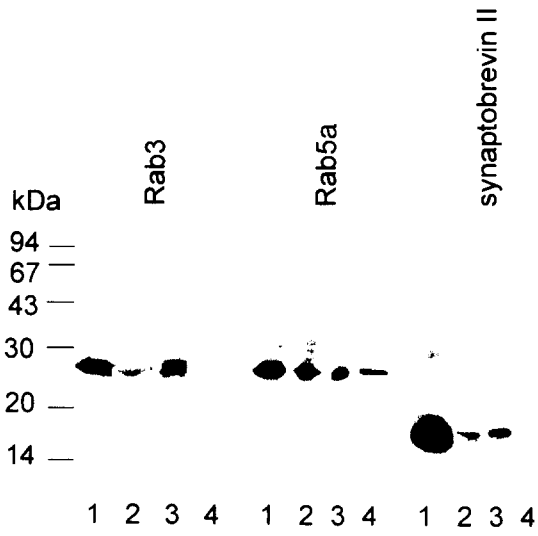

Fig. 3. Enrichment of Rab3-, Rab 5- and synaptobrevin II-like proteins during the course of purification of AQP-CD-containing vesicles. Proteins were analyzed by SDS PAGE/immunoblotting with the indicated monoclonal antibodies against Rab3 (Cl 42.1), Rab 5a (Cl 621.2) and synaptobrevin II (Cl 69.1) and with ^{125}I anti mouse IgG antibody. Shown are autoradiograms of filters. The following preparations were analyzed: lanes 1, rat brain (30 μg of protein); lanes 2, homogenate from rat kidney medulla/papilla (60 μg of protein), lanes 3, AQP-CD-containing vesicles from rat kidney medulla/papilla (60 μg of protein); lanes 4, membranes from L cells (60 μg of protein).

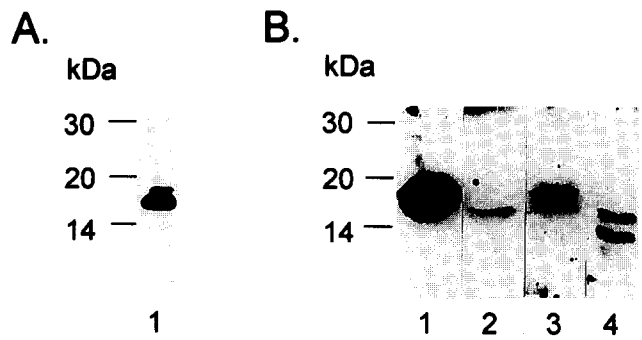

Fig. 4. Detection of proteins by a monoclonal antibody recognizing synaptobrevin I, synaptobrevin II and cellubrevin in preparations from rat brain, rat renal medulla/papilla and L cells. Proteins were analyzed by SDS PAGE/immunoblotting with the monoclonal antibody Cl 10.1. Filter-bound antibodies were visualized with the ECL system. (A) Rat brain membranes (30 μg of protein) were employed. The exposure time was 15 s. (B) The following preparations were analyzed: lane 1, rat brain membranes (30 μg of protein); lane 2, homogenate from rat kidney medulla/papilla (60 μg of protein); lane 3, AQP-CD-containing vesicles from rat kidney medulla/papilla (60 μg of protein); lane 4, membranes from L cells (60 μg of protein). The exposure time was three minutes (compare with the exposure time in panel A).

The detection of Rab3- and synaptobrevin II-like proteins in the vesicle preparation and their enrichment during the course of purification of AQP-CD-containing vesicles is of particular interest. Rab3 proteins in neuronal and neuroendocrine cells appear to be crucially involved in the fusion of secretory vesicles with the plasma membrane [26]. The identified Rab3-like protein may serve a similar function in kidney epithelial cells. In analogy to the intracellular localization and role of synaptobrevins in neuronal cells, the synaptobrevin II-like protein in the kidney may be a part of the essential docking/fusion machinery of AQP-CD-containing, regulated vesicles. The absence of synaptotagmin I and synaptophysin, both implicated in Ca^{2+}-dependent exocytosis [16,17,27], is consistent with a cAMP-triggered, Ca^{2+}-independent exocytosis in renal epithelial cells. In neurons Rab3a requires a presumably Ca^{2+}-binding protein, rabphilin 3a, to be linked to Ca^{2+}-triggered exocytosis [28]. It is intriguing to speculate that principal cells express a rabphilin 3a homolog regulated by cAMP/PKA.

Rab5 proteins are involved in the fusion of early endosomes and vesicle recycling [11], whereas proteins of the Rab3 family

have been implicated in exocytosis. Recent data demonstrate an overlap between Rab5 or Rab3a-containing compartments and point to a stage-specific association of Rab5 and Rab3a proteins with synaptic vesicles [13]. Hence, the detection of both Rab3- and Rab5a-like proteins in our preparation suggests that the purification yields either (a) AQP-CD-containing vesicles from different parts of a recycling compartment, carrying one or the other protein, or (b) vesicles carrying both. The observation that – in contrast to Rab3 – Rab5a is not enriched with AQP-CD does not preclude the presence of Rab5a on AQP-CD containing vesicles, as we cannot distinguish between the different stages of recycling vesicles. Similar considerations may apply to the vesicular distribution of cellubrevin, a component of the ubiquitous regulated and constitutive pathways of endocytosing and recycling receptors [22].

The present report provides structural evidence that vasopressin-induced antidiuresis is similar to regulated exocytosis in neuronal and non-neuronal secretory cells. The identification of Rab3-, Rab5a- and synaptobrevin II-like proteins in the kidney may be a first step towards understanding the molecular mechanisms underlying the antidiuretic action of vasopressin.

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