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AQP2 is a substrate for endogenous PP2B activity within an inner medullary AKAP-signaling complex

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Jo, Inho, Donald T. Ward, Michelle A. Baum, John D. Scott, Vincent M. Coghlan, Timothy G. Hammond, and H. William Harris. AQP2 is a substrate for endogenous PP2B activity within an inner medullary AKAP-signaling complex. *Am J Physiol Renal Physiol* 281: F958–F965, 2001.—We have demonstrated that inner medullary collecting duct (IMCD) heavy endosomes purified from rat kidney IMCD contain the type II protein kinase A (PKA) regulatory subunit (RII), protein phosphatase (PP)2B, PKCζ, and an RII-binding protein (relative molecular mass ~90 kDa) representing a putative A kinase anchoring protein (AKAP). Affinity chromatography of detergent-solubilized endosomes on cAMP-agarose permits recovery of a protein complex consisting of the 90-kDa AKAP, RII, PP2B, and PKCζ. With the use of small-particle flow cytometry, RII and PKCζ were localized to an identical population of endosomes, suggesting that these proteins are components of an endosomal multiprotein complex. ^{32}P-labeled aquaporin-2 (AQP2) present in these PKA-phosphorylated endosomes was dephosphorylated in vitro by either addition of exogenous PP2B or by an endogenous endosomal phosphatase that was inhibited by the PP2B inhibitors EDTA and the cyclophilin-cyclosporin A complex. We conclude that IMCD heavy endosomes possess an AKAP multiprotein-signaling complex similar to that described previously in hippocampal neurons. This signaling complex potentially mediates the phosphorylation of AQP2 to regulate its trafficking into the IMCD apical membrane. In addition, the PP2B component of the AKAP-signaling complex could also dephosphorylate AQP2 in vivo.

kidney; water channel; adenosine 3′,5′-cyclic monophosphate; protein phosphatase 2B; aquaporin-2; A kinase anchoring protein

TRANSEPITHELIAL WATER REABSORPTION across renal collecting duct cells is stimulated by arginine vasopressin (AVP). AVP acts by increasing the number of aquaporin-2 (AQP2) water channels in the apical membrane of inner medullary collecting ducts (IMCDs), thus increasing its permeability to water (reviewed in Refs. 19 and 27). Before AVP stimulation, AQP2 resides primarily in subapical membrane vesicles, and the subsequent stimulation increases the insertion of these AQP2-containing vesicles into the apical membrane itself. It is known that AVP binding to the V_2 receptor stimulates cAMP formation and that the insertion of AQP2 vesicles into the apical membrane involves phosphorylation of AQP2 by cAMP-dependent protein kinase A (PKA). A present area of active investigation is how the activated PKA interacts with its AQP2 substrate and whether it is specifically targeted to it. In addition, although a number of studies have investigated PKA-mediated AQP2 phosphorylation, little is known regarding the dephosphorylation of AQP2.

The COOH-terminal region of AQP2 contains a single putative phosphorylation site for PKA (Ser^{256}) (12, 26). In LLC-PK$_1$ cells transfected with wild-type AQP2, addition of AVP and forskolin induces insertion of AQP2-containing vesicles into the cell membrane. However, when such expression studies are performed with an AQP2 mutant possessing an alanine instead of Ser^{256}, this AQP2 insertion response is abolished (16). Studies using both purified AQP2-containing vesicles (22) and *Xenopus laevis* oocytes (21) suggest that PKA-mediated phosphorylation of AQP2 does not alter its intrinsic water permeability but rather may be a key event permitting AQP2 access to the plasma membrane. Recently, Klussmann et al. (20) have provided evidence for the involvement of A kinase anchoring proteins (AKAPs) in the translocation of AQP2 from intracellular vesicles to the IMCD membrane. Although these data demonstrate that compartmentalization of PKA by AKAPs is necessary for AQP2 translocation, questions remain regarding the structure of this PKA-AKAP complex and the location of the intracellular compartment to which it is bound.

The subcellular trafficking of vesicles has been studied extensively in neurons, which are also polarized

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and may be related to epithelial cells (17). The postsynaptic densities of hippocampal neurons have been demonstrated to contain a multiprotein-signaling complex consisting of a 79-kDa A kinase anchoring protein (150 kDa in mice) (7) associated with a number of signaling molecules, including the PKA regulatory subunit, protein phosphatase 2B (PP2B or calcineurin), and PKC (reviewed in Ref. 8). This hippocampal neuron-signaling complex is postulated to provide the molecular specificity for cycles of PKA-mediated phosphorylation and dephosphorylation of membrane protein components, including ion channels. In mice, a novel AKAP family has also been cloned that localizes to the apical membranes of renal proximal tubule cells (AKAP-KL) (9).

Given the postulated evolutionary and functional relationships between polarized neurons and epithelial cells (17), we investigated whether rat IMCD heavy endosomes, which we have previously shown to be apically derived and AQP2 containing (14, 22), also contain such an AKAP complex, because such a mechanism could help explain how AQP2 trafficking is regulated in response to AVP. Furthermore, given the presence of PP2B in the neuronal AKAP complex, we investigated whether it is also expressed in IMCD cells and whether it can dephosphorylate AQP2 previously phosphorylated by PKA.

METHODS

Materials. Various items were obtained from the following sources: [γ-^{32}P]ATP (10 Ci/mmol) was from NEN Life Science Products, Sepharose 4B was from Pharmacia (Uppsala, Sweden), polyvinylidene fluoride membrane was from MSI (Westborough, MA), and SDS, acrylamide, β-mercaptoethanol, and ammonium persulfate were from Bio-Rad. All other reagents were from either sources described previously (22) or Sigma (St. Louis, MO). Male Sprague-Dawley rats (200–250 g) were purchased from Charles River Laboratories (Cambridge, MA).

Purification of IMCD endosomes. Endosomes were prepared from rat kidney inner medullary papillae as described previously (14). Briefly, papillae were homogenized in *buffer A* (300 mM mannitol and 12 mM HEPES, titrated to pH 7.6 with Tris) and centrifuged at 2,500 *g* to remove nuclei and unlysed cells. The postnuclear supernatant was centrifuged at 20,000 *g* for 20 min, and the new supernatant and the lightly packed upper layer of the pellet were collected. This postmitochondrial supernatant was then recentrifuged at 48,000 *g* for 30 min, and the resulting pellet was resuspended in *buffer A* and fractionated on a self-forming Percoll gradient (18% Percoll by weight). After centrifugation for 30 min at 48,000 *g*, the bottom one-third of the gradient was collected and dispersed in an eightfold larger volume of *buffer B* [(in mM) 300 mannitol, 100 KCl, 5 $MgSO_4$, and 5 HEPES, adjusted to pH 7.6 with Tris] and left on ice for 15 min. This vesicle suspension was then recentrifuged at 48,000 *g* for 30 min, and the resulting membrane pellet was resuspended in *buffer B*. After centrifugation at 5,000 *g* for 15 min, the endosomes appeared as a pearly white layer overlaying a darker membrane pellet. These vesicles, previously characterized as apical endosomes, were either used immediately or stored at −80°C until use. Endosomal protein content was determined by the method of Bradford (3).

Immunoblotting. Western blot analysis of IMCD proteins was performed as described previously (15) using the following antisera: anti-PKCζ and anti-PKCδ rabbit polyclonal (Life Technologies, Gaithersburg, MD), and, anti-RII goat polyclonal and anti-PP2B (catalytic subunit) mouse monoclonal antibodies (Upstate Biotechnology, Lake Placid, NY).

Immunohistochemistry. Rats were perfusion fixed as described previously (25), using freshly prepared 4% paraformaldehyde followed by sucrose cryoprotection. Tissue samples were then embedded in OCT compound (Miles, Elkart, IN), snap-frozen in 2-methylbutane in liquid N_2, and stored at −70°C until further use. Immunohistochemistry was then performed as previously described (24) using frozen sections (4 μm) cut from the tip of individual rat kidney inner medullae and stained with various antisera. Counterstaining was performed with methyl green (Fisher, Pittsburgh, PA).

^{32}P-RII overlay. Rat tissue samples were subjected to SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane that was incubated in buffer containing 1% (wt/vol) bovine serum albumin and recombinant RII protein, which was phosphorylated using [γ-^{32}P]ATP as described previously (7). The membrane was incubated in the resulting ^{32}P-RII probe for 14 h at 4°C in the presence or absence of the blocking peptide Ht31 (4). The membrane was then washed free of unbound probe, and the presence of bound ^{32}P-RII was determined by autoradiography.

Affinity chromatography using cAMP-agarose. IMCD heavy endosomes were solubilized on ice for 2 h in hypotonic buffer [(mM) 10 HEPES (pH 7.9), 1.5 $MgCl_2$, 10 KCl, 1 polymethylsulfonyl fluoride, 0.5 dithiothreitol, 1 benzamidine, and 0.01 IBMX] containing 0.5% (vol/vol) Nonidet P-40 and centrifuged at 15,000 *g* for 15 min. The detergent-soluble supernatant was mixed with cAMP-agarose equilibrated in hypotonic buffer containing 0.1% Nonidet P-40. After being mixed by rotation at 4°C for 14 h, the cAMP-agarose pellet was washed four times in hypotonic buffer and then analyzed for its protein content by SDS-PAGE and immunoblotting (6).

Localization of dual antibody labels by small-particle flow cytometry. Freshly prepared endosomes were incubated in 1:1 normal donkey serum (clarified by centrifugation at 180,000 *g* for 20 min) for 1 h at room temperature, washed, and then incubated in primary antibodies produced in goats or rabbits at 4°C for 14 h. The endosomes were washed again and incubated in fluorescently conjugated anti-donkey secondary antibody (1:100 dilutions) for 2 h at room temperature. After a final wash, endosomes were analyzed by small-particle flow cytometry (14). Secondary antibodies (Jackson Immunochemicals, Bar Harbor, ME) were donkey anti-goat cyanine-5 (for anti-RII) and donkey anti-rabbit cyanine-3 (for anti-AQP2 and anti-PKCζ). Cyanine-3 was excited with 100 mW of 488-nm blue argon ion laser light, and cyanine-5 was excited with 100 mW of 647-nm ruby-red krypton laser light. The fluorescence of each of the 2,000 individual particles was collected in photomultipliers beyond a 575 ± 26- or 675 ± 20-nm (cyanine-3 or cyanine-5, respectively) band-pass filter. Flow cytometry files were collected and analyzed using Becton Dickinson Cell Quest flow cytometry software.

[^{32}P] labeling and immunoprecipitation of AQP2. Endosomes were phosphorylated using the catalytic subunit of PKA in the presence of [γ-^{32}P]ATP, as described previously (22). The ^{32}P-labeled endosomes were then subjected to various treatments during incubation at 37°C in buffer containing (in mM) 20 Tris (pH 7.0), 50 KCl, 3 Mg^{2+}, 0.1 Ca^{2+}, 0.5 Ni^{2+}, and 0.1 dithiothreitol as well as 3,900 U PKI. The resulting [^{32}P]AQP2 was then collected by immunoprecipitation and assayed for its ^{32}P content. Briefly, after ^{32}P labeling, endosomes were solubilized using 1% (vol/vol) Triton

X-100 (1 h on ice) and centrifuged at 14,000 *g* for 30 min. The supernatant was combined with rabbit anti-AQP2 antiserum coupled to Sepharose 4B and incubated with continuous mixing at 4°C for 14 h. The immunoprecipitate was pelleted by centrifugation, washed three times in phosphate-buffered saline, and denatured with Laemmli buffer. [^{32}P]AQP2 was then resolved by SDS-PAGE, detected by autoradiography, and quantified by densitometry.

RESULTS

Purified IMCD heavy endosomes possess a 90-kDa AKAP, RII, PP2B, and PKCζ. With the use of specific antisera, Fig. 1 shows that rat inner medulla contains a 55-kDa RII species and the 18-kDa PP2B β-subunit (Fig. 1, *A* and *B*). Sands et al. (25) have reported previously that purified rat IMCD heavy endosomes also possess PKCζ. Because these three proteins represent the basic components of an AKAP-signaling complex present in hippocampal neurons (10, 18), we sought to determine whether IMCD heavy endosomes also possess a similar AKAP-signaling complex. As detected using a ^{32}P-RII overlay assay shown in Fig. 1, AQP2 endosomes also possess AKAP(s). To demonstrate that the RII probe is binding specifically to endogenous AKAP, we used the anchoring inhibitor peptide Ht31 to ablate its binding (Fig. 1*C*, *lane 6*). Ht31 represents the peptide sequence within AKAP that is responsible for RII binding (8). We have assigned the single most prominent band a molecular mass of ~90 kDa based on data from multiple experiments (n = 12). However, it should be noted that the band is broad and in some experiments appears as a doublet that included a smaller band of an ~75-kDa relative molecular mass (Fig. 1*C*). More complete characterization will be required to determine whether these multiple AKAP bands observed on ^{32}P-RII overlay blots result from either proteolysis of a larger AKAP protein or to posttranslational modification of an AKAP(s). Alternatively, these multiple bands may derive from two independent species of RII-binding protein. The data shown in Fig. 1 are consistent with and extend those reported by Klussman et al. (20).

To verify that RII, PP2B, and PKCζ colocalized within collecting duct cells, we immunostained sections of rat inner medulla using antisera specific for each of the proteins. As shown in Fig. 2, rat IMCDs exhibited diffuse immunoreactivity to each of the antibodies tested with the exception of anti-PP2B antibody, which strongly stained the apical region of IMCD cells (Fig. 2*C*).

IMCD heavy endosomes possess a multiprotein phosphorylation complex similar to that found in neurons. With the employment of a methodology similar to that used previously to demonstrate the existence of an AKAP-signaling complex in neurons (7), detergent-solubilized IMCD heavy endosomes were mixed with cAMP-agarose, with the resulting cAMP-agarose-

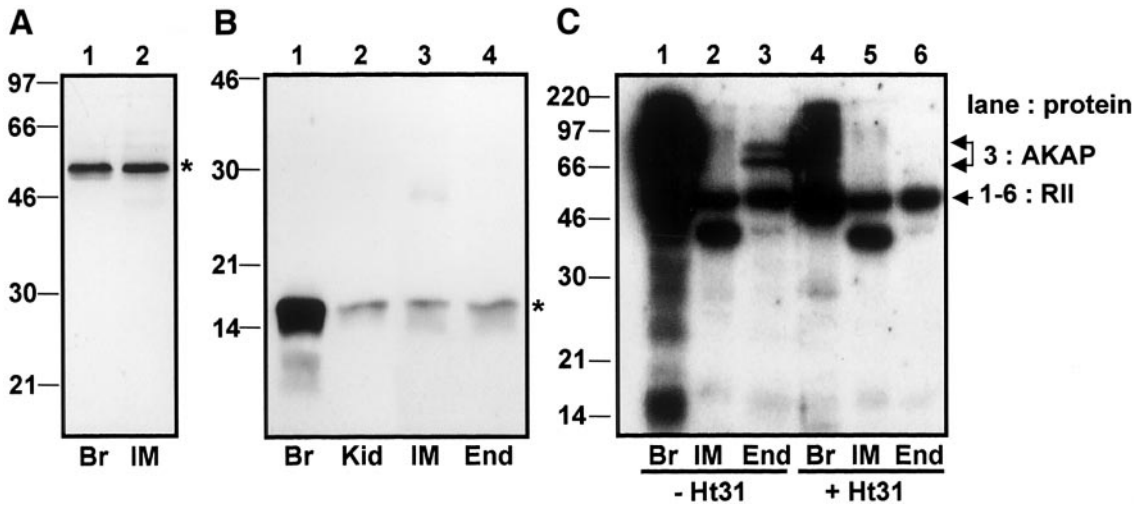

Fig. 1. Presence of type II protein kinase A (PKA) regulatory subunit (RII), protein phosphatase (PP)2B, and a putative A kinase anchoring protein (AKAP) in rat inner medulla (IM). *A*: rat IM homogenate (40 μg protein) contains a single 55-kDa anti-RII immunoreactive band that comigrates on immunoblots with a similar band present in rat brain (Br), demonstrating that rat IM contains type II PKA. *B*: immunoblot showing that total rat kidney homogenate (Kid), total IM homogenate, and IM endosomes (End) each contains a PP2B-immunoreactive band that comigrates with neuronal PP2B. *C*: autoradiograph showing the binding of [^{32}P]-labeled RII to proteins from rat brain (*lanes 1* and *4*), IM membranes (*lanes 2* and *5*), and inner medullary collecting duct (IMCD) endosomes (*lanes 3* and *6*; 40 μg protein/lane) resolved by SDS-PAGE and electrophoretically transferred to a membrane. Incubation of the membrane with ^{32}P-RII was performed in the absence (*lanes 1–3*) or presence (*lanes 4–6*) of Ht31 anchoring inhibitor peptide. When the ^{32}P-RII probe binds to a protein and the binding is inhibited by Ht31, the association indicates the presence of a putative AKAP (4). The rat brain sample exhibits numerous specific associations and is overexposed due to the large quantities of RII-binding proteins compared with kidney. In contrast, purified aquaporin-2 (AQP2)-containing endosomes contain 2 specific RII-binding proteins (*right*). Note that the binding of the ^{32}P-RII probe to a 55-kDa protein is present in all 3 tissues and was not affected by the presence of Ht31. This binding results from dimerization of the ^{32}P-RII probe with RII in the sample. *Left*: positions of the molecular mass markers. Results are representative of a minimum of 4 separate experiments.

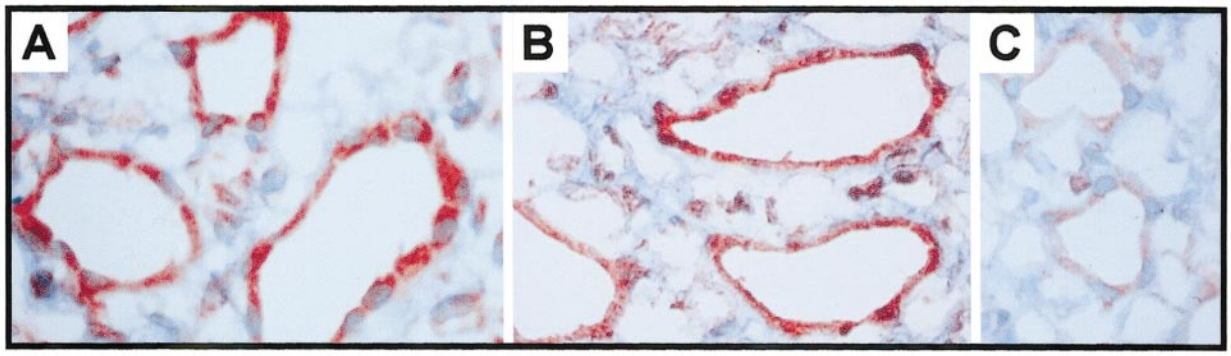

Fig. 2. Rat inner medullary collecting ducts contain RII, PP2B, and PKCζ. Representative light microscopy immunohistochemical stains show that IMCDs contain RII (*A*), PP2B (*B*), and PKCζ (*C*) and that the antibody staining for each protein includes immunoreactivity in the region of the apical membrane. Specific antibody binding is indicated by the rose-colored reaction product (magnification: 2,500).

bound proteins identified by an anti-RII immunoblot or by a ^{32}P-RII overlay assay. As shown in Fig. 3*A*, cAMP-agarose retained both RII and the 90-kDa putative AKAP, substantially clearing these proteins from a mixture of detergent-soluble endosomal proteins.

By employing affinity chromatography using cAMP-agarose, we then examined whether any endosomal proteins can be coabsorbed with RII and the 90-kDa putative AKAP and, if so, whether they represent other components of the hippocampal AKAP complex such as PP2B and PKC. As shown in Fig. 3*B*, the resulting cAMP-agarose precipitate contained RII (*lane 1*), a 90-kDa AKAP (*lane 2*), PP2B (*lane 4*), and PKCζ (*lane 5*). Anti-PKCδ (*lane 6*) and anti-AQP2 (*lane 7*) antisera failed to immunodetect either protein in this complex despite these proteins being abundantly present in purified IMCD heavy endosomes (25).

Colocalization of RII with AQP2 and PKCζ in the same IMCD endosomal population. To further test the hypothesis that RII and PKCζ are associated together within a putative AKAP complex present in IMCD heavy endosomes, small-particle flow cytometry was utilized to examine whether anti-RII and anti-PKCζ antibodies colocalize to the same population of heavy endosomes. Rat IMCD heavy endosomes, shown previously to contain abundant AQP2 (14), were incubated with antisera against RII and PKCζ, and their presence was determined with species-specific secondary

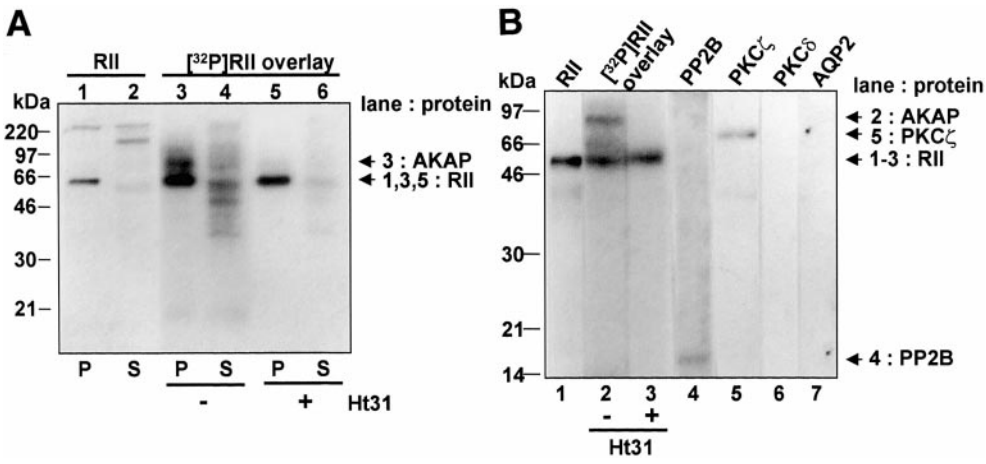

Fig. 3. Absorption of AKAP-containing multiprotein complex from IMCD endosomes using cAMP-agarose. *A*: cAMP-binding proteins and their associated proteins were affinity precipitated from Nonidet (N)P-40-solubilized purified AQP2 containing IMCD endosomes using cAMP-agarose. The cAMP-agarose precipitate was pelleted by centrifugation and solubilized in SDS-Laemmli buffer. The pellet (P) and supernatant (S) were assayed for their RII content by immunoblotting (*lanes 1* and *2*) and for their AKAP content by ^{32}P-RII overlay assay (*lanes 3–6*). The cAMP-agarose successfully precipitated almost all of the RII and the putative 90-kDa AKAP broad band contained in IMCD endosomes. *B*: a multiprotein complex was subsequently purified from NP-40-solubilized AQP2 endosomes by affinity chromatography using cAMP-agarose. Equal amounts of the cAMP-agarose precipitate were loaded onto consecutive lanes of an SDS-PAGE gel, and the proteins were electrophoretically transferred to a membrane. Individual lanes of the membrane were then separated, and each was either immunoblotted against a specific antiserum (*lanes 1* and *4–7*) or incubated in ^{32}P-RII in the absence (*lane 2*) or presence (*lane 3*) of Ht31 blocking peptide and developed by autoradiography. As shown here, the cAMP-agarose precipitate contains RII (*lane 1*), a 90-kDa AKAP (*lane 2*), PP2B (*lane 4*), and PKCζ (*lane 5*) but not PKCδ (*lane 6*) or AQP2 (*lane 7*). *Left*: positions of the molecular mass markers. Results are from a minimum of 4 independent experiments.

antibodies coupled to either of the fluorophores cyanine-3 or cyanine-5. Perfusion of the rats with fluorescein-dextran (F-dextran) before kidney harvest results in the uptake of the concentrated F-dextran by endocytic vesicles retrieved from the apical membrane of the IMCD as previously described (14). Therefore, through the measurement of fluorescence at the three different emission wavelengths corresponding to excitation of fluorescein, cyanine-3, and cyanine-5, it is possible to demonstrate whether a single F-dextran-containing endosome possesses one or two specific proteins bound to its cytoplasmic surface.

Exposure of purified IMCD endosomes to both secondary antisera conjugated with either cyanine-3 or cyanine-5 yielded an overall low level of cyanine-3- or -5-fluorescent endosomes (Fig. 4*A*). This control demonstrates there is little nonspecific fluorophore binding in the absence of primary antibodies. As an additional control, the same endosomes were incubated with an irrelevant control rabbit polyclonal antiserum that also produced little (5%) highly fluorescent cyanine-3- and -5-binding endosomes (Fig. 4*B*). In contrast, preincubation of F-dextran-containing endosomes with the combination of rabbit anti-PKCζ (*x*-axis) and goat anti-RII (*y*-axis) antisera produced a significant number (40%) of highly fluorescent endosomes. In a similar manner, coincubation of endosomes with rabbit anti-AQP2 (*x*-axis) and goat anti-RII (*y*-axis) also produced a group (27%) of F-dextran-containing endosomes containing bound anti-AQP2 and RII antibodies (Fig. 4*D*). Taken together, these data suggest that components of an AKAP complex are present on the surface of F-dextran-containing endocytic vesicles. It is not possible to perform similar flow cytometry studies on vesicles

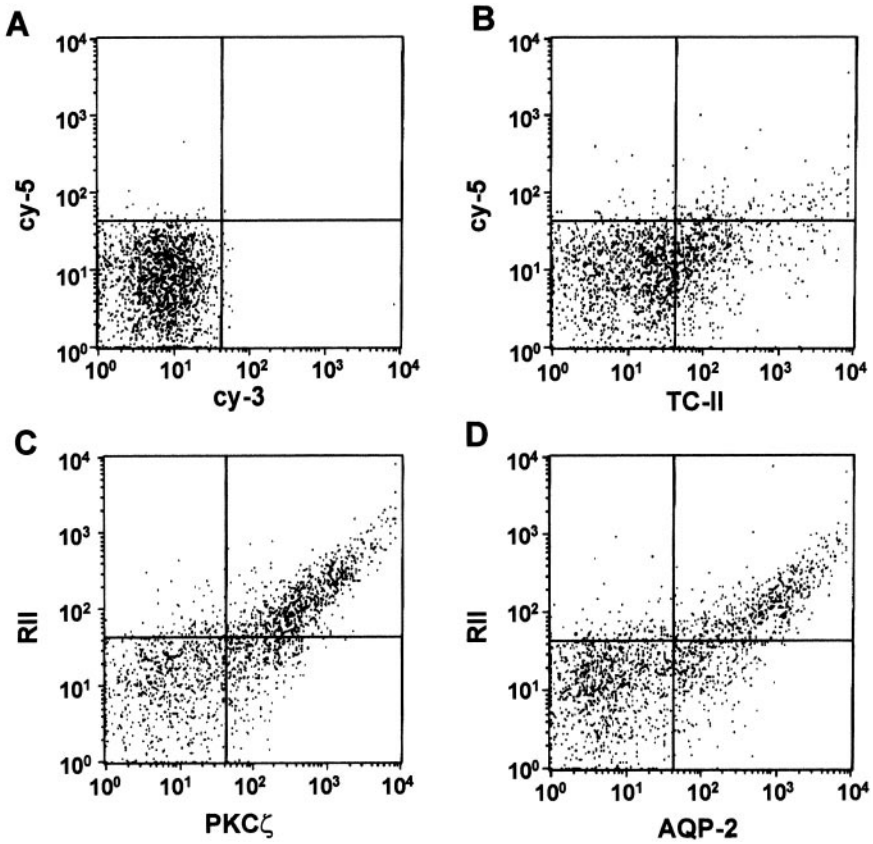

Fig. 4. Colocalization of RII with AQP2 and PKCζ in the same IMCD endosomal population. Two-dimensional plots show the colocalization of various antisera on fluorescein-dextran (F-dextran)-containing rat IMCD endosomes as detected by small-particle flow cytometry. Each diagram shows 2,000 individual endosomes containing F-dextran entrapped within their lumens as a frequency histogram displayed on an arbitrary log scale. The *x*-axis displays binding of donkey anti-rabbit cyanine-3 (cy-3), the *y*-axis shows binding of donkey anti-goat cyanine-5 (cy-5), and the origin of each of the axes is marked by a zero. In *A*, there is little nonspecific binding of secondary antisera to endosomes in the absence of primary antibodies. On the basis of these data, an arbitrary value equal to 40 times basal fluorescence in either cyanine-3 or -5 was established as the threshold gating of signal shown in the box in the *top right* corner. Addition of an irrelevant rabbit antiserum yields a similar result, albeit with larger background fluorescence (5%). In contrast, incubation with rabbit anti-PKCζ (*x*-axis) and goat anti-RII (*y*-axis) antisera yields 40% of particles that are highly cofluorescent for both cyanine-3 and -5 (*C*, *top right* quadrant). Thus because both antibodies bind to endosomes, this indicates colocalization of the two proteins in the same population of F-dextran-containing endosomes. In *D* (*top right* quadrant), there is colocalization of AQP2 (*x*-axis) and RII (*y*-axis) in the same endosomes (27% highly fluorescent). It is noteworthy that vesicles or membranes not containing entrapped F-dextran are not visualized by this method. Results are from F-dextran fluorescent vesicles derived from aliquots of a single vesicle preparation per experiment that was performed a total of 5 times.

immunoisolated using anti-AQP2 antiserum (23) because of an inability to elute the resulting bound vesicles without loss of the F-dextran fluorophore that is essential for flow cytometry detection, as shown in Fig. 4 (data not shown).

AQP2 is dephosphorylated by endogenous PP2B activity and by exogenous PP2B in vitro. Previously, we have shown that the AQP2 present in IMCD heavy endosomes is phosphorylated by endogenous PKA activity (22). To test whether this endogenous AQP2 is also a substrate for exogenous PP2B in vitro, endosomes were ^{32}P labeled using the catalytic subunit of PKA and incubated at 37°C for various intervals in the presence or absence of an exogenous PP2B catalytic subunit. AQP2 was then immunoprecipitated from the endosomes, and the ^{32}P content of AQP2 was determined by SDS-PAGE and autoradiography. As shown in Fig. 5, addition of the PP2B catalytic subunit increased the rate of [^{32}P]AQP2 dephosphorylation.

To examine whether the endogenous phosphatase activity that dephosphorylates AQP2 in vitro is reduced by addition of inhibitors of PP2B, the ^{32}P-labeled endosomes were subjected to various incubations in the presence or absence of PP2B inhibitors. Incubation of ^{32}P-labeled endosomes at 37°C resulted in a time-dependent reduction in the ^{32}P content of AQP2 (Fig. 6, *lanes 1–4*). Coincubation with the cyclophilin-cyclosporin A complex inhibited this dephosphorylation by 50% (±13 SD, $n = 6$, *lane 5*). Exposure of endosomes to EDTA completely abolished AQP2 dephosphorylation (*lane 6*). AQP2 dephosphorylation was also inhibited by incubating the endosomes on ice (*lane 7*), suggesting that the phosphatase responsible is sensitive to temperature and divalent cation chelation as well as the cyclophilin-cyclosporin A complex. To demonstrate that incubation on ice does not permanently inactivate this phosphatase, another aliquot of ^{32}P-labeled endosomes was incubated on ice and then at 37°C to demonstrate that AQP2 dephosphorylation still occurred (*lane 8*).

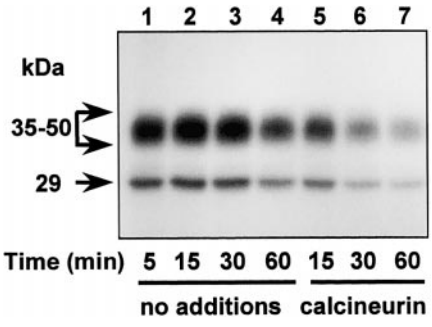

Fig. 5. Dephosphorylation of [^{32}P]AQP2 in IMCD endosomes is increased by exogenous PP2B. ^{32}P-labeled AQP2-containing endosomes were incubated at 37°C for either 5 (*lane 1*, control), 15 (*lanes 2* and *5*), 30 (lanes *3* and 6), or 60 min (*lanes 4* and *7*) with either no additions (*lanes 1–4*) or in the presence of 0.1 μg/μl PP2B (*lanes 5–7*). [^{32}P]AQP2 was then immunoprecipitated from each of the samples as described in METHODS and detected by autoradiography. *Left*: positions of the nonglycosylated (29-kDa) and glycosylated (35- to 50-kDa) AQP2 species.

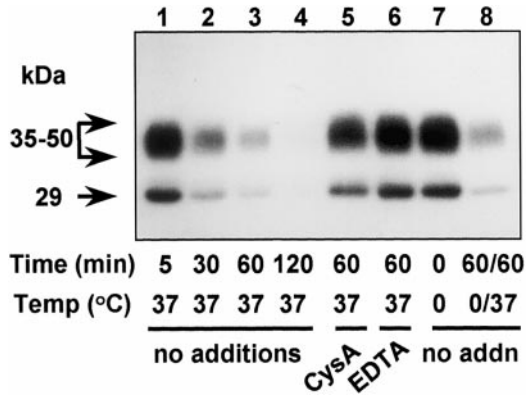

Fig. 6. Endogenous PP2B mediates [^{32}P]AQP2 dephosphorylation in IMCD endosomes. ^{32}P-labeled AQP2-containing endosomes were incubated at 37°C for either 5 (*lane 1*, control), 30 (*lane 2*), 60 (*lane 3*), or 120 min (*lanes 4–6*) with either no additions (*lanes 1–4*) or in the presence of 1 mM cyclophilin-10 μM cyclosporin A complex (CysA; *lane 5*) or 5 mM EDTA (*lane 6*). Alternatively, the ^{32}P-labeled endosomes were incubated for 60 min on ice (*lane 7*) or for 60 min on ice followed by 60 min at 37°C (*lane 8*). [^{32}P]AQP2 was then detected as in Fig. 5.

DISCUSSION

The phosphorylation of AQP2 by PKA is a crucial signal in the AVP-elicited water reabsorption pathway. The trafficking of AQP2 vesicles into the IMCD apical membrane is regulated by PKA activity (reviewed in Ref. 27) and AKAPs (19), although the exact subcellular location of the PKA responsible for AQP2 phosphorylation has not been determined. Studies of the postsynaptic densities of hippocampal neurons have revealed the existence of a signaling complex containing RII, PP2B, and PKC that is bound to a 79-kDa AKAP. This multiprotein complex permits the regulation of membrane receptors and ion transporters, such as AMPA/kainate receptors, Ca^{2+} channels, or *N*-methyl-D-aspartate receptors, via protein phosphorylation by PKA or PKC or via dephosphorylation by PP2B (11).

The data reported here show that a purified population of IMCD heavy endosomes also contains the various protein components of a similar AKAP complex. In previous work, these IMCD heavy endosomes have been shown to *1*) be of apical origin, *2*) possess abundant AQP2 protein by immunoblotting analysis, and *3*) exhibit a very large mercury-sensitive osmotic water permeability as determined by using stop-flow fluorimetry (14, 22). A combination of Western blotting and immunocytochemistry suggests that AKAP complex constituents, including RII, PP2B and PKCζ, are present in IMCD tubules.

Studies using a ^{32}P-RII probe demonstrate its binding to a protein band/doublet of 75-to 90-kDa approximate molecular mass in purified IMCD heavy endosomes (Fig. 3). The 75- to 90-kDa putative AKAP protein(s) reported on here awaits further characterization to determine whether it is a novel member of the AKAP family or represents a member of the AKAP-KL (expressed in kidney and lung) family of proteins, as reported by Dong et al. (9). Although AKAP-KL

has been localized to the apical membranes of murine proximal tubules, it has not been reported as being expressed in IMCD.

Affinity chromatography using cAMP-agarose demonstrated that RII, PP2B, PKCζ, and a single 90-kDa RII-binding protein (putative AKAP) were all retained on the cAMP matrix whereas PKCδ and AQP2 were not absorbed. Data reported here suggest the presence of a multiprotein AKAP-signaling complex, similar to that described in neurons, in rat AQP2 apical endosomes. Because renal AQP2 expression is upregulated in dehydration and after chronic AVP treatment, it would be interesting to know whether these conditions also alter the relative expression of these AKAP complex proteins.

To demonstrate that these proteins are actually associated with apical endosomes rather than being present in contaminating membranes in some portion of the purified IMCD heavy endosomal preparation, we performed three-color, small-particle flow cytometry to colocalize constituents of a putative AKAP complex on the surface of apical endosomes containing entrapped F-dextran. The studies demonstrate that binding of both RII and PKCζ is colocalized on the cytoplasmic surface of F-dextran-containing vesicles, providing further evidence for the presence of an AKAP complex in apically derived IMCD endosomes. A significant number of these endosomes also contain immunoreactive AQP2 protein.

The data displayed in Figs. 1–4 provide the first evidence for the presence of an AKAP complex on the endocytic arm of AQP2-containing vesicles in IMCD cells. In contrast, studies by Klussmann et al. (20) provide strong support for the importance of AKAPs in the corresponding exocytic arm of AQP2 vesicle insertion into the IMCD apical membrane. Thus the presence of AKAPs in AQP2-containing vesicles may be similar to the situation in neurons, where the AKAP-signaling complex is a dynamic structure with successive phases of protein association and disassociation. It is possible that the AKAP-containing signaling complex described here would possess all the kinases and phosphatases necessary to potentially initiate and terminate the AVP-mediated insertion and removal of AQP2 water channels in the IMCD apical membrane. A similar mechanism might also mediate the function of other membrane transporter proteins. In this regard, ROMK1 channels expressed in *Xenopus laevis* oocytes are insensitive to forskolin treatment, but when ROMK1 is coexpressed with the 79-kDa AKAP, it exhibits cAMP-dependent activation (1).

We reported previously (22) that AQP2 can be both phosphorylated by an endogenous cAMP-dependent protein kinase and dephosphorylated by an endogenous phosphatase present in IMCD heavy endosomes. Data presented here extend these studies and show that endosomal [^{32}P]AQP2 is dephosphorylated by an endogenous phosphatase that is inhibitable by EDTA or the cyclophilin-cyclosporin A complex. Moreover, AQP2 dephosphorylation also occurred after addition of the exogenous PP2B catalytic subunit (Fig. 5). Together, these data indicate that PKA-phosphorylated AQP2 is an in vitro substrate for PP2B and suggest that PP2B may also be present on the cytoplasmic surfaces of IMCD heavy endosomes in vivo. However, additional work is necessary to demonstrate whether AQP2 dephosphorylation will promote its retrieval from the apical IMCD membrane. Moreover, the incomplete inhibition of endogenous AQP2 dephosphorylation by the cyclophilin-cyclosporin A complex might also suggest the presence of another divalent cation-dependent phosphatase in IMCD heavy endosomes.

The functional role of PKCζ in the endosomal signaling complex is not understood. In this regard, rat AQP2 contains a PKC phosphorylation consensus sequence at Ser^{226} (Ser^{231} in human AQP2). Although there is evidence to suggest that PKC activation may have an inhibitory effect on AVP-induced water reabsorption in collecting ducts (2), it is not known whether this effect is caused by the PKC-mediated phosphorylation of AQP2 as occurs with AQP4 (13). Alternatively, PKC may act indirectly, perhaps promoting retrieval of AQP2 endosomes in a process similar to that described for glucose transporters expressed in oocytes (28). The evidence linking PKC to water reabsorption is presently based on the use of phorbol esters (1,2 diacylglycerol analogs) that are unable to activate the atypical PKCζ, whereas PKCζ is instead known to be activated via phosphatidylinositol 3-kinase. Thus, if the PKCζ present within this AKAP-signaling complex does play a role in water reabsorption, then the role could be to modulate the cAMP-mediated ADH response by a phosphatidylinositol 3-kinase-mediated hormonal or ionic signal.

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