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Geurt Stokman, Yu Qin, Hans-Gottfried Genieser, Frank Schwede, Emile de Heer, Johannes L. Bos, Ingeborg M. Bajema, Bob van de Water and Leo S. Price

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cAMP Stimulates Apical Exocytosis of the Renal Na⁺-K⁺-2Cl⁻ Cotransporter NKCC2 in the Thick Ascending Limb : ROLE OF PROTEIN KINASE A

Paulo S. Caceres, Gustavo R. Ares and Pablo A. Ortiz

J. Biol. Chem., September 11, 2009; 284 (37): 24965-24971.

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Epac Regulates UT-A1 to Increase Urea Transport in Inner Medullary Collecting Ducts

Yanhua Wang, Janet D. Klein, Mitsi A. Blount, Christopher F. Martin, Kimilia J. Kent, Vladimir Pech, Susan M. Wall and Jeff M. Sands

JASN, September, 2009; 20 (9): 2018-2024.

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Renal expression of exchange protein directly activated by cAMP (Epac) 1 and 2

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Li Y, Konings IB, Zhao J, Price LS, de Heer E, Deen PM. Renal expression of exchange protein directly activated by cAMP (Epac) 1 and 2. *Am J Physiol Renal Physiol* 295: F525–F533, 2008. First published May 21, 2008; doi:10.1152/ajprenal.00448.2007.—In the kidney, many physiological processes of ion transport and cellular proliferation are mediated via cAMP, which classically activates protein kinase A (PKA). Recently, however, two new cAMP targets, the exchange protein directly activated by cAMP (Epac) 1 and 2, were identified, which mediate alternative pathways to PKA. To investigate their renal expression, antibodies specifically recognizing Epac1 and Epac2 were generated and used in rat immunohistochemistry with antibodies recognizing aquaporin-1 (AQP1), Tamm-Horsfall protein, Calbindin-D_{28K}, and AQP2 to mark proximal tubules (PT)/thin descending limbs of Henle's loop (tDLH), thick ascending limbs of Henle's loop (TAL), distal convoluted tubule/connecting tubule (DCT/CNT), and the collecting duct (CD) principal cells, respectively. Epac1 and Epac2 were expressed at the brush border of PT cells but were absent from tDLH cells. In the TAL, Epac1 and Epac2 were expressed throughout the cells with some confinement toward the apical membrane. In the DCT/CNT, Epac1 was confined to the apical region of the cells, whereas Epac2 was mainly expressed in the apical and basolateral regions. In the CD, a dispersed Epac1 expression was found in intercalated cells only (cortical CD), principal and intercalated cells [outer medullary CD (OMCD)], and mainly AQP2-negative cells in the inner medullary CD (IMCD). In contrast, Epac2 expression was at the apical and basolateral membrane of cortical principal cells, dispersed and apical in the OMCD, and in all cells of the IMCD. A similar distribution for Epac1/2 was found in the human kidney. The observed expression in different tubular segments suggests a major role for Epac 1/2 in tubular transport physiology and cellular proliferation.

kidney; localization

CYCLIC ADENOSINE MONOPHOSPHATE (cAMP) is a common second messenger controlling many cellular processes. Protein kinase A (PKA) is a general acceptor for cAMP, resulting in the phosphorylation of a large variety of cellular targets. A newly described cAMP target, exchange protein directly activated by cAMP (Epac) 1, and its close relative Epac2 contain cAMP-binding domains very similar to the cAMP-binding domains in the regulatory subunit of PKA and activate specifically the monomeric G proteins of Ras family, Rap1 and Ras (6, 22). Many actions of cAMP, which were thought to be mediated exclusively by PKA, are now found to be likely mediated through Epac, including integrin-mediated cell adhesion (29), vascular endothelial cell barrier formation (13, 25), cardiac gap

junction formation (32), mitogen-activated protein kinase (MAPK) signaling (34), hormone gene expression (14), and phospholipase C- ϵ (PLC- ϵ) activation (16, 30). Intriguingly, newly published findings demonstrate that Epac-mediated actions of cAMP influence Na⁺, K⁺, Ca²⁺, and Cl[−] channel function (1, 15, 20), Na⁺/H⁺ and Na⁺/K⁺ transporter activity, and exocytosis in multiple cell types (17, 19, 37).

Interestingly, the kidney is one of the organs that shows the highest levels of expression of mRNAs encoding Epac1 (6), especially in the collecting ducts, suggesting an important role of Epac in cAMP signaling mechanisms in the kidney. Therefore, to identify which renal segments might involve Epac1 or 2 in their cellular processes, we investigated the localization of Epac1 and 2 in the rat kidney.

MATERIALS AND METHODS

Cell culture. Human embryonic kidney 293 (HEK293) cells were grown in DMEM supplemented with 10% (vol/vol) fetal calf serum at 37°C in 5% CO₂ and transiently transfected with the empty expression vector pcDNA3, or those containing Flag-tag full-length human Epac1 (GI: 3978530) cDNA or Flag-tag full-length mouse Epac2 (GI: 9790086) cDNA using polyethylenimine (Polysciences). HEK293 cells were seeded on polylysine-coated glass slides in 12-well plate (Corning Costar, Cambridge, MA) at a density of 1×10^5 cells/well. The medium was refreshed and the cells were transfected 3 h later. The transfection mix for each well consisted of 1 μ g cDNA and 6 μ l polyethylenimine (1 μ g/ μ l) in a total volume of 30 μ l Opti-MEM (Invitrogen). It was incubated for 20 min at room temperature and added to the cells. After an overnight incubation at 37°C, the medium was refreshed and the cells were grown for 2–3 days.

Tissue preparation. Male Wistar rats were obtained from the University Animal Facility, Radboud University Nijmegen Medical Centre. Following anesthesia with isofluorothane, the rats were killed after which the kidneys were rapidly removed, fixed in 1% (wt/vol) periodate-lysine-paraformaldehyde (PFA) for 2 h and 15% (wt/vol) sucrose in PBS for 16 h, fast frozen in liquid nitrogen and stored at −80°C.

Human donor kidneys were provided by Eurotransplant. These kidneys had not been transplanted for technical reasons (branching of the renal artery), but showed no histological abnormalities. The warm ischemia time of these kidneys was 88 min, and the cold ischemia time was 32 h. These kidneys did not show significant signs of ischemia-reperfusion injury (2, 3). All samples were obtained after informed consent of the donors. Their use was approved by the medical ethical committee of Eurotransplant. Kidney cortex and medulla samples were fixed in 4% buffered formalin and embedded in paraffin.

Antibodies. For Epac detection, a cDNA fragment encoding human Epac1 (GI: 3978530) except for amino acids 1–148 (Δ DEP-Epac1)

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and a cDNA fragment encoding mouse Epac2 (GI: 9790086) except for amino acids 1–279 (Δ DEP-Epac2) were cloned into the pGEX expression vector to produce recombinant proteins. Rabbits were immunized with the GST-coupled Δ DEP-Epac1 or Δ DEP-Epac2 recombinant proteins and, after development of proper antibody titres, the rabbits were bled. Polyclonal rabbit anti-serum 2293 was used to detect Epac1; rabbit polyclonal anti-serum 2295 was used to detect Epac2. Preimmune sera were used as negative controls. Mouse monoclonal anti-Flag antibody (M2; Sigma, St. Louis, MO) was used to detect the Flag-Epac proteins in transfected HEK293 cells.

As primary antibodies against renal segment marker proteins, we used mouse monoclonal aquaporin-1 (AQP1) antibodies (kindly provided by Dr. Jennings, University of Texas, Galveston, TX) to detect proximal tubules (PT) and thin descending limbs of Henle's loop (tDLH), polyclonal goat Tamm-Horsfall protein (THP) antibodies (ICN Biomedicals, Aurora, OH) to reveal the thick ascending limbs of Henle's loop (TAL), mouse monoclonal Calbindin-D_{28K} antibodies (Swant, Bellinzona, Switzerland) to detect the distal convoluted tubule (DCT) and connecting tubule (CNT), and affinity-purified guinea pig 9 AQP2 antibodies, raised against the last 15 amino acids of the AQP2 COOH terminus (7) to reveal the principal cells of the collecting duct. Mouse monoclonal anti- β -actin antibodies were from Sigma.

As secondary antibodies, Alexa 594-conjugated goat anti-mouse, donkey anti-goat, or goat anti-guinea pig antibodies were used to detect AQP1, THP, Calbindin-D_{28K}, and AQP2. Alexa 488-conjugated goat anti-rabbit were used to detect Epac1 and 2 on the kidney sections. All secondary antibodies were purchased from Molecular Probes (Leiden, The Netherlands).

Immunoblotting. Transfected HEK293 cells from 12-well plate were lysed in 200 μ l Laemmli buffer/well and 15- μ l samples were analyzed. PAGE, blotting, and blocking of the PVDF membranes were done as described (18). Membranes were incubated for 16 h with 1:1,000 diluted polyclonal rabbit anti-Epac1 or Epac2 antibodies, or 1:1,000 diluted monoclonal mouse anti-Flag antibody, in Tris-buffered saline Tween 20 (TBS-T) supplemented with 5% nonfat dried milk. Blots were incubated for 1 h with 1:5,000 diluted goat anti-rabbit IgGs or 1:2,000 goat anti-mouse IgGs (Sigma) as secondary antibodies coupled to horseradish peroxidase. Proteins were visualized using enhanced chemiluminescence (ECL, Pierce, Rockford, IL). Equal loading of the samples was confirmed by parallel immunoblotting against 1:25,000 diluted mouse β -actin antibody.

Immunocytochemistry and immunohistochemistry. For immunocytochemistry, transfected HEK293 cells were washed twice with PBS-CM (PBS with 0.1 mM CaCl₂, 1 mM MgCl₂) and fixed in 3% paraformaldehyde for 30 min. Following quenching of aldehyde groups with 50 mM NH₄Cl in PBS for 15 min, cells were permeabilized with 0.3% Triton X-100 in PBS for 15 min, incubated with goat serum dilution buffer (GSDB; 16% goat serum, 0.3% Triton X-100, 0.3 M NaCl in PBS) for 30 min to block nonspecific antibody binding, and incubated O/N with polyclonal rabbit anti-Epac1 or 2 antibody at 1:100 dilution in GSDB. After being washed twice with permeabilization buffer (0.3% Triton X-100, 0.1% BSA in PBS), cells were incubated with 1:100 diluted goat anti-rabbit antibodies coupled to Alexa 488 in GSDB for 45 min. Next, cells were rinsed twice with permeabilization buffer and mounted on glass slides with Mowiol.

For immunohistochemistry on 7- μ m cryosection of the rat kidney, the following protocol was used. 1) For antigen retrieval, the sections were immersed in boiled citrate target retrieval buffer [0.01 M sodium citrate and 0.01 M citric acid (pH 6.0)] and left to cool for 30 min. 2) To block nonspecific antibody binding, sections were incubated in TN-B buffer [TN: 0.1 M Tris-HCl (pH 7.6), 0.15 M NaCl; B: 0.5% Blocking Reagent, Perkin Elmer Life Sciences, Boston, MA] for 30 min. 3) Sections were incubated O/N at 4°C with 1:100 diluted Epac1 or Epac2 antibodies or their preimmune sera. 4) Following three times of 10-min washing with TN-T buffer (TN buffer with 0.05% Tween 20), the sections were incubated for 1 h at room temperature with Alexa 488-conjugated goat anti-rabbit antibodies. 5) After being

washed, the sections were incubated with mouse AQP1 antibodies (1:100), goat Tamm-Horsfall antibodies (1:200), mouse Calbindin-D_{28K} antibodies (1:100), or guinea pig AQP2 antibodies (1:50) for 1 h at room temperature, followed by Alexa 594-conjugated goat anti-mouse, donkey anti-goat, or goat anti-guinea pig antibodies for 1 h at room temperature. 6) Sections were dehydrated and mounted in Mowiol. Images were made by using Bio-Rad MRC-1024 confocal laser-scanning microscopy (CLSM).

For the human kidneys, paraffin sections of 4- μ m thickness were cut, deparaffinized, and rehydrated. For Epac1 staining, antigen retrieval was achieved by boiling sections in 10 mM Tris/1 mM EDTA buffer at pH 8.0 for 10 min, followed by 20 min of cooling. For Epac2 staining, antigen retrieval was achieved by boiling sections in 10 mM citrate buffer at pH 6.0 for 10 min, followed by 20 min of cooling. Endogenous peroxidase was blocked by 0.3% H₂O₂. Subsequently, the sections were incubated with rabbit anti-Epac1 (1:300), anti-Epac2 (1:150), or anti-AQP2 (1:50) for 1 h. After being rinsed, the sections were incubated with anti-rabbit-Envision or anti-guinea pig-Envision (Dako, Glostrup, Denmark), developed with diaminobenzidine and Cu₂SO₄, and counterstained with hematoxylin. Subsequently, the sections were dehydrated using increasing ethanol concentrations, xylene, and mounted in malinol.

To check for the specificity of the anti-Epac1 and 2 immunostaining on the rat sections, the Epac antibodies at the concentrations given above were incubated O/N at 4°C with their antigenic GST- Δ DEP-Epac1 (15 mg/ml) or GST- Δ DEP-Epac2 (5 mg/ml) recombinant proteins before application. For the human sections, the anti-Epac1 and anti-Epac2 antibodies were first serially diluted until the next one in two dilution step resulted in reduction of immunostaining. The dilution of anti-Epac antibodies was incubated with a 10-fold molar excess of either Epac1 or Epac2 recombinant protein for 4 h at 4°C followed by immunostaining of normal human kidney paraffin sections. Control sections were incubated with unabsorbed antibodies at the same dilution.

RESULTS

Specificity of the Epac1 and 2 antibodies. To test the specificity of rabbit polyclonal antibodies 2293 and 2295 for Epac1 and 2, respectively, HEK293 cells were transiently transfected with F-Epac1 or F-Epac2 expression constructs. After 3 days, the cells were fixed and subjected to immunocytochemistry using these antibodies. CLSM revealed a clear staining with the Epac1 antibodies in cells expressing F-Epac1, but not F-Epac2 (Fig. 1A, *top*). Moreover, the Epac2 antibodies only stained F-Epac2, but not F-Epac1, transfected cells (Fig. 1A, *bottom*). In parallel, cells were scraped and lysed to test our antibodies with immunoblotting. As shown in Fig. 1B, our Epac1 antibodies specifically recognized a band at 96 kDa in the lane loaded with F-Epac1 transfected cell material, which is the anticipated size for F-Epac1. No signal was found in the lanes loaded with samples derived from mock or F-Epac2 expressing cells (Fig. 1B). In contrast, our Epac2 antibodies recognized a 116-kDa band in the lane loaded with F-Epac2 expressing material, which is in line with the anticipated mass of F-Epac2. No such signals were obtained in lanes loaded with lysates from mock or F-Epac1 expressing cells. Immunoblotting with Flag antibody recognized the same bands for Epac1 and 2 in the cells. Similar β -actin signals confirmed loading of protein equivalents in each lane (Fig. 1B). These data indicated that our rabbit polyclonal antibodies against Epac1 and Epac2 specifically recognize Epac1 and Epac2, respectively, when used in immunocytochemistry and in immunoblotting.

Fig. 1. Specificity of the Epac1 and 2 antibodies. **A:** F-Epac1 or F-Epac2 expressing human embryonic kidney 293 (HEK293) cells were immunostained with rabbit Epac1 or 2 antibodies, followed by Alexa 488-conjugated goat-anti-rabbit secondary antibody. The cells were analyzed by confocal laser-scanning microscopy (CLSM). Images are shown with a 180-fold magnification. **B:** lysates of mock-transfected or F-Epac1 or F-Epac2 expressing HEK293 cells were separated by gel electrophoresis and immunoblotted for Epac1, Epac2, or the Flag tag, whereas immunoblotting for β -actin was done to check equal loading. **C:** rat kidneys were fixed with 3% paraformaldehyde (PFA), cryosections were made and incubated with rabbit Epac1 or Epac2 antibodies, or rabbit preimmune serum, followed by Alexa 488-conjugated goat-anti-rabbit secondary antibody. **D:** consecutive cryosections were immunostained as in **C** with the rabbit Epac1 or 2 antibodies, or the antibodies were preabsorbed with their antigenic recombinant proteins. Images are shown with a 60-fold magnification. Arrows indicate the tubules of inspection.

To determine whether our Epac antibodies reveal specific signals in the rat kidney, neighbouring sections were incubated with 1:100 dilutions of these sera or their respective preimmune sera. Subsequent CLSM analysis revealed clear signals with the anti-Epac1 antibodies and anti-Epac2 antibodies (Fig. 1C, *left*, arrows indicating positively stained renal tubules), which were not seen with their respective preimmune sera (Fig. 1C, *right*, arrows indicating the same tubules as those in the

left). These data indicated that both antibodies reveal specific Epac signals in the rat kidney. Furthermore, preabsorption of the antibodies with their antigenic recombinant proteins fully blocked the fluorescent signal (Fig. 1D, arrows indicating the same tubules for both *left* and *right*), demonstrating the specificity of these antibodies.

Localization of Epac1 in rat kidney. To elucidate the renal segmental localization of Epac1 and 2, coimmunohistochemi-

Fig. 2. Expression of Epac1 in the rat kidney. Renal sections were doublelabeled for Epac1 (green) and aquaporin-1 (AQP1; red, A–C), Tamm-Horsfall protein (THP; red, D), Calbindin-D_{28K} (red, E), or AQP2 (red, F–H) and analyzed by CLSM. Images are shown with a 10- and 60-fold magnification. Arrows indicate the tubules of inspection. Bold arrows indicate collecting duct (CD) principal cells, and arrowheads indicate CD intercalated cells.

Fig. 3. Expression of Epac2 in the rat kidney. Renal sections were doublelabeled for Epac 2 (green) and AQP1 (red, A–C), THP (red, D), Calbindin-D_{28K} (red, E), or AQP2 (red, F–H) and analyzed by CLSM. Images are shown with a 10-, 30-, 60-, 120-, and 180-fold magnification. Arrows indicate the tubules of inspection and apical membrane of the tubular cells, whereas double arrows indicate basolateral membrane. Arrowheads indicate connecting tubule (CNT) intercalated cells.

cal staining of rat kidney sections for Epac1 or 2 with nephron segment marker proteins was performed. PT and tDLH express AQP1, the TAL expresses THP, the DCT and CNT express Calbindin-D_{28K}, and principal cells of the collecting duct (CD) express AQP2.

Double labeling of Epac1 with AQP1 revealed a colocalization in ~30% of the AQP1-positive PT in cortex (Fig. 2A), but

no expression of Epac1 in AQP1-positive segments of the outer medulla (Fig. 2B). Interestingly, in all tubules stained with Epac1, the signals were predominantly confined to the brush-border membrane region of the cells (Fig. 2A, arrows). In the inner medulla, AQP1-positive tDLH tubules did not show Epac1-specific staining. Colabeling with THP showed that Epac1 was expressed in all TAL cells (Fig. 2D). In these cells,

Fig. 4. Immunohistochemistry of Epac1 and Epac2 in the human kidney. *A*: human renal sections were labeled by indirect immunoperoxidase for Epac1 and Epac2 or the antibodies preincubated with their antigenic recombinant proteins. Magnification $\times 10$. *B*: human kidney sections of cortex and medulla were immunolabeled as in *A*. Arrows indicate apical localization of Epac1 and 2 on distal tubular epithelium (left) and double arrows on CD (right). Bold arrows indicate cytoplasmic Epac2 in proximal epithelium and arrowheads in glomerular visceral epithelium (left bottom). Magnification $\times 20$. *C*: sequential human kidney sections were immunolabeled as in *A* with Epac2 and AQP2 antibodies. Arrowheads indicate CD intercalated cells. Magnification $\times 100$.

Epac1 expression was spread throughout the cells, with a somewhat stronger staining at the apical side of the cells (Fig. 2D, arrows). In the DCT and CNT, Epac1 was found in all Calbindin-D_{28K}-positive cells at only the apical region of the cells, but its expression was clearly weaker than in the PT (Fig. 2E, arrows). In the CDs, Epac1 expression was found all along the tubule (Fig. 2, F–H; bold arrows indicate principal cells and arrowheads intercalated cells). In the cortex, Epac1 was found in intercalated cells, in which it was evenly distributed. In the outer medulla, Epac1 was mainly expressed in intercalated and to some extent in (AQP2-positive) principal cells. In the inner medulla, Epac1 was again mainly found in AQP2-negative cells.

Localization of Epac2 in rat kidney. Double labeling of Epac2 with AQP1 revealed a clear Epac2 expression in PT of the cortex and outer medulla (Fig. 3, A and B; arrows). Its expression was somewhat dispersed over the cell, but was mainly localized at the brush-border side and sometimes also at the basolateral side of the cells (double arrows). The identity of the Epac2-positive structures in the lumen of the PT is unclear. The AQP1-positive, tDLH-positive tubules of inner medulla did not show staining for Epac2 (Fig. 3C).

Similar to Epac1, extensive expression of Epac2 was found in the TAL as all THP-positive cells stained for Epac2 (Fig. 3D). Moreover, it had a dispersed vesicular-like expression pattern and confinement to the apical side of the cells (arrows). Epac2 was also found in the DCT and CNT, because it was expressed in all Calbindin-D_{28K}-positive tubular cells and intercalated cells (Fig. 3C, arrows and arrowheads). In these cells, Epac2 was found in the apical (arrow) and basolateral (double arrow) region of the cells. In the CD, all AQP2-positive cells stained for Epac2 (Fig. 3, F–H, arrows). In all segments, Epac2 was also mainly found at apical regions of the cells, with some expression throughout and at the basolateral side of both intercalated and principal cells.

Localization of Epac1 and Epac2 in the human kidney. To determine the localization of Epac1 and 2 in the human kidney, it was first tested whether the antibodies would yield specific staining. Indeed, following preabsorption of the antibodies with their antigenic recombinant proteins, a complete absence (Epac1) or a marked reduction (Epac2) of Epac1 and Epac2 staining on human kidney was observed (Fig. 4A). However, virtually no inhibition of staining was observed, when anti-Epac1 antibodies were incubated with Epac2, or vice versa (not shown). The subsequent immunoperoxidase staining using our Epac antibodies showed that the localization of Epac1 and Epac2 was essentially similar to that in the rat kidney (Fig. 4B). Epac1 was found in both proximal and distal epithelial cells, TAL, and CD with a predominant apical distribution. Glomerular epithelium only showed a weak staining. Peritubular capillaries and glomerular endothelium were negative. Epac2 staining was observed in a similar apical distribution in renal epithelia, but in addition showed some staining in visceral epithelial cells in glomeruli. To investigate whether Epac2 in the CD localized to principal or intercalated cells, consecutive staining of Epac2 and AQP2 was performed (Fig. 4C). Similar to that in the rat kidney, Epac2-positive staining was observed in both AQP2-positive (principal) and AQP2-negative (intercalated; double arrowheads) cells in the CDs, with a predominant expression to the apical membrane.

DISCUSSION

During the last years, an alternative pathway for cAMP signaling has emerged with the identification of Epac1 and 2. Considering the important role that this cascade plays in the regulation of several different processes in the kidney, we set out to determine the Epac1 and Epac2 protein expression in the rat and human kidney. For this, we used rabbit polyclonal antibodies 2293 and 2295, which were generated against human Epac1 and mouse Epac2, respectively. As shown in Fig. 1, these antibodies specifically recognized Epac1 or 2 proteins without cross-reacting to each other. Furthermore, they also showed signals in the rat kidney, which were Epac-specific because neither the preimmune sera nor the anti-sera after preabsorption showed positive staining throughout the kidney. Immunostaining of both Epac isoforms in human and rat kidneys showed similar protein distribution.

Epac in the PT. Our immunohistochemistry revealed Epac1 and Epac2 expression throughout the nephron and CD (Fig. 5). In line with the detected high expression of Epac1 mRNA (26)

Fig. 5. Summary of Epac1 and 2 localization in the kidney tubular cells. Epac1 and 2 localizations are listed in renal segments and cell types for rat (A) and human (B). G, glomerular cells; PT, proximal tubule cells of cortex (c) or outer medulla (o); tDLH, thin descending limb of Henle's loop cells; TAL, thick ascending limb of Henle's loop cells; DCT/CNT, distal convoluted tubular and connecting tubular cells; CCD, cortical collecting duct cells; OMCD, outer medullary collecting duct cells; IMCD, inner medullary collecting duct cells; mCD, medullary collecting duct cells; none, no staining observed.

and the supposed functional implication of Epac1 in the cAMP-mediated regulation of the Na^+/H^+ exchanger 3 (NHE3) in the brush border (17, 26) of mouse PT, we found a clear expression of Epac1 in the rat PT (Fig. 2A). The observed pattern for Epac1 (and Epac2) appeared similar as found for megalin, which is expressed in all three PT segments, but mainly segments 1 and 2 (5). However, as the functional effects on NHE3 were deduced from the response to an Epac-specific agonist, and both Epac1 and Epac2 are found in the apical region of PT (Figs. 2A and 3, A–B), the observed effect might have also been mediated by Epac2. Interestingly, ANG II has been shown to stimulate collagen synthesis in mouse mesangial cells through a novel Epac-mediated phosphoinositide 3-kinase-dependent pathway (36). In the kidney, ANG II increases sodium and bicarbonate reabsorption in the apical membrane of the PT via AT_1 receptors, in which Epac1 or 2 might also be involved. The structures in the center of the tubules were seen for both Epac1 and 2 staining, although more often for Epac2. At present, it is unclear to us whether these are erythrocytes and/or tubular cells. Some of these structures showed as cells with a round shape and are disconnected from the tubules around them. These may be erythrocytes, as these have been reported to express Epac (28). Others are more irregular, are often connected with the tubules around them, and may therefore be tubular microvilli.

Epac in the TAL. In the TAL, vasopressin induces apical sodium entry by increasing the expression and activation of the $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporter (NKCC2) and activation of the $\text{Na}^+-\text{K}^+-\text{ATPase}$ located in the basolateral membrane (23, 36). Whereas activation of the sodium pump is ascribed to PKA-mediated phosphorylation of its α -subunit (33), Epac1 and Epac2 might have a role in this process, as both are highly expressed throughout this nephron segment (Figs. 2D and 3D).

Epac in the DCT and CNT. In the DCT and CNT, several transport processes, such as the adenosine-induced uptake of magnesium and TRPV5-mediated calcium reabsorption, are known to be regulated by cAMP (8, 21). Both Epac1 and Epac2 are found in these nephron segments with the confinement at the cell membrane (Figs. 2E and 3E). It has to be established whether they have a role in these processes.

Epac in the CD. In the kidney CD, several processes have been suggested to involve Epac. In microdissected rat CCD, Laroche-Joubert et al. (26) demonstrated that the $\text{H}^+-\text{K}^+-\text{ATPase}$ is activated through a cAMP-Epac1-Rap1-Raf-B-ERK cascade in $\text{I}\alpha$ intercalated cells. Involvement of Epac1, however, was again deduced from using an Epac1/2-specific agonist. As we find expression of both Epac proteins in these cells (Figs. 2F and 3F), our data suggest that the observed effect could be mediated by Epac1 and/or Epac2.

Following incubation of rat inner medulla sections with the Epac agonist 8-pCPT-2'-O-Me-cAMP, Yip (15, 38) found that Epac is involved in the vasopressin-induced Ca^{2+} mobilization and translocation of AQP2 toward the apical membrane. Based on immunocytochemical detection using only Epac1-specific antibodies, Yip suggested that Epac1 mediates this response in the inner medullary CD (IMCD). In line with our data (Fig. 2H), however, close examination of the particular figure of Yip et al. reveals that his IMCD Epac1 expression is also mainly confined to AQP2-negative cells. Although an involvement of Epac1 cannot be excluded at this stage, the complete overlap of Epac2 and AQP2 expression in the IMCD cells (Fig. 3H) may

suggest that Epac2, rather than Epac1, mediates the vasopressin-induced calcium mobilization and AQP2 translocation in the IMCD.

Interestingly, several data suggest that sodium reabsorption through the epithelial sodium channel ENaC, which is expressed in the CNT and CD, might also involve Epac1 and 2. It has been shown that in rat pulmonary cells, dopamine increases the channel open probability (P_o) of ENaC by a cAMP-mediated alternative signaling pathway involving Epac and Rap1 (27). Moreover, in Madin-Darby canine kidney cells stably expressing ENaC, cAMP has been shown to increase the short-circuit current (I_{SC}) by increasing the surface expression of ENaC, which is thought to occur through phosphorylation of ENaC (31). In *Xenopus laevis* oocytes, however, the stimulation by cAMP is abolished with the expression of recombinant ENaC mutated at ERK phosphorylation motifs, but not of PKA phosphorylation sites (24), which may suggest that cAMP stimulates ENaC functioning through an Epac/ERK-mediated phosphorylation of ENaC.

Epac in proliferation. Besides roles in mediating signaling cascades regulating ion channels and transporters, the role of Epac in cellular proliferation has also been suggested through its activation of Rap1 in various organisms (6, 9–12). Interestingly, several hormones, such as ANG II, AVP, and endothelin, induce cellular renal proliferation through phosphorylation events. Moreover, cellular proliferation is a hallmark for polycystic kidney disease and lithium-induced NDI, disorders of (mostly) the CD in which cAMP has a critical role (4, 35). It remains to be established, however, whether Epac has a critical role in these physiological and pathophysiological processes of cellular proliferation.

In conclusion, we established the renal localization of the two Epac isoforms, 1 and 2. While the particular function of each of these isoforms awaits further investigation, our data provide a basis for further studies toward the elucidation of the role of PKA or Epac proteins in cAMP-mediated processes of ion transport and cellular proliferation in the kidney.

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