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Regulation of the expression of the Cl^-/anion exchanger pendrin in mouse kidney by acid-base status

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Regulation of the expression of the Cl^-/anion exchanger pendrin in mouse kidney by acid-base status.

Background. Pendrin belongs to a superfamily of Cl^-/anion exchangers and is expressed in the inner ear, the thyroid gland, and the kidney. In humans, mutations in pendrin cause Pendred syndrome characterized by sensorineural deafness and goiter. Recently pendrin has been localized to the apical side of non-type A intercalated cells of the cortical collecting duct, and reduced bicarbonate secretion was demonstrated in a pendrin knockout mouse model. To investigate a possible role of pendrin in modulating acid-base transport in the cortical collecting duct, we examined the regulation of expression of pendrin by acid-base status in mouse kidney.

Methods. Mice were treated orally either with an acid or bicarbonate load (0.28 mol/L NH_4Cl or $NaHCO_3$) or received a K^+-deficient diet for one week. Immunohistochemistry and Western blotting was performed.

Results. Acid-loading caused a reduction in pendrin protein expression levels within one day and decreased expression to 23% of control levels after one week. Concomitantly, pendrin protein was shifted from the apical membrane to the cytosol, and the relative abundance of pendrin positive cells declined. Similarly, in chronic K^+-depletion, known to elicit a metabolic alkalosis, pendrin protein levels decreased and pendrin expression was shifted to an intracellular pool with the relative number of pendrin positive cells reduced. In contrast, following oral bicarbonate loading pendrin was found exclusively in the apical membrane and the relative number of pendrin positive cells increased.

Conclusions. These results are in agreement with a potential role of pendrin in bicarbonate secretion and regulation of acid-base transport in the cortical collecting duct.

The Cl^-/anion exchanger pendrin (SLC26A4) belongs to a superfamily of anion exchangers currently consisting of at least nine identified mammalian members (SLC-26A1-9) [1–6]. Significant pendrin expression is found only in the thyroid gland, the inner ear, the endometrium and the kidney [7–9]. Mutations in pendrin cause Pendred syndrome (OMIM # 27400) characterized by sensorineural deafness and goiter [10, 11]. Pendrin is expressed on the apical membrane of thyrocytes and may function there as a chloride/iodide exchanger supplying iodide for thyroid hormone synthesis [12]. In the inner ear the function of pendrin is unknown at present. In the kidney, expression of pendrin was first reported in the rat cortex and medulla with positive immunohistochemical staining of proximal tubules [13]; however, subsequent studies by other groups in mouse, rat, and human kidney found expression only in the cortex, in the distal tubule and cortical collecting duct [2, 7, 8]. In the collecting duct pendrin was localized to the apical membrane of intercalated cells that were negative for the A cell-specific kidney isoform of the band 3 protein kAE1 [8].

Based on functional studies and immunohistochemistry, at least two subtypes of intercalated cells in the cortical collecting duct have been described: type A and type B cells [14–16]. Type A cells participate in the generation of bicarbonate by secreting H^+ through V-type H^+-ATPases across the apical membrane and releasing bicarbonate through the basolateral band 3-like kAE1 Cl^-/HCO_3^- exchanger. In contrast, B cells secrete bicarbonate across the apical membrane and release H^+ through basolateral V-type H^+-ATPases [14, 15, 17]. While the molecular nature of the apical bicarbonate transport system has not been completely clarified to date, two candidate proteins have been suggested: pendrin and anion exchanger 4 (AE4) [8, 18]. In addition, some authors discuss the existence of a third subtype of intercalated cells, the so-called non-A/B-cell or γ-cell [19–21]. Functionally this subtype of intercalated cell has been described as having DIDS-insensitive Cl^-/HCO_3^- exchange on both the apical and basolateral side [19]. Morphologically, this type of cell has been characterized both by the absence of AE1 and the apical expression of an H^+-

Key words: bicarbonate secretion, chloride anion exchanger, sensorineural deafness, goiter, cortical collecting duct, metabolic alkalosis, base transport.

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ATPase [20, 21]. However, no molecular marker has been found to date that positively identifies this cell type.

In pendrin knockout mice reduced bicarbonate secretion by the cortical collecting duct in metabolic alkalosis was recently demonstrated, suggesting a role for pendrin in apical bicarbonate secretion by B intercalated cells [8]. However, patients suffering from Pendred syndrome have not been reported to possess a renal defect (see OMIM and [10, 11]).

The present study was performed to investigate the regulation of pendrin expression and localization in mouse kidney in response to changes in acid-base status in order to understand the role of pendrin in modulating acid-base transport along the cortical collecting duct. We demonstrate that pendrin expression is strongly reduced by either acid intake or a K^+-deficient diet and that both treatments lead to a cellular redistribution of pendrin to the cytosol. During a metabolic alkalosis pendrin was localized exclusively to the apical membrane. Therefore, these data are consistent with the potential role of pendrin in mediating base transport in the cortical collecting duct.

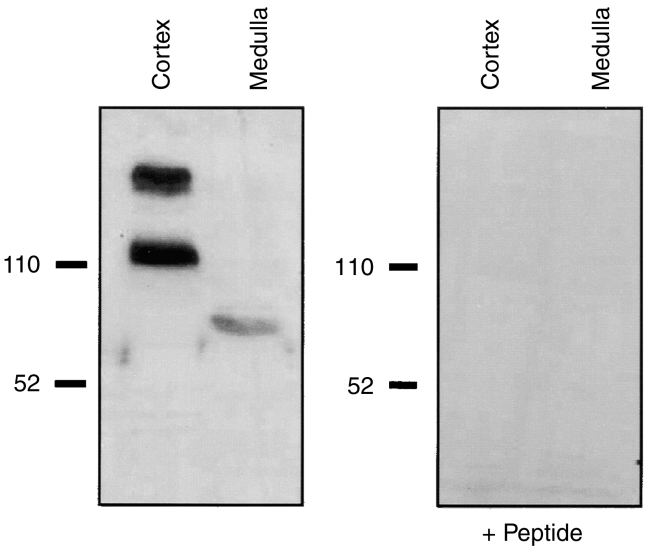

Fig. 1. Expression of pendrin in mouse renal cortex and medulla. (*A*) The antibody against pendrin recognized two bands of about 110 and 220 kD in Western blotting of a crude membrane preparation of dissected mouse kidney cortex. No such bands were detected in a medullary membrane fraction from the same mouse kidney. (*B*) Same membrane as in (A) after stripping and reprobing with the anti-pendrin antibody after preincubation with the immunizing peptide. No bands were detected.

METHODS

Animal studies

C57BL/6J mice (male, 25 to 30 g; Jackson Laboratory, Westgrove, PA, USA) were maintained on standard chow and had access to drinking water ad libitum. Mice were given either 2% sucrose/0.28 mol/L NH_4Cl or 2% sucrose/0.28 mol/L $NaHCO_3$ to the drinking water for 24 hours, 48 hours, or 7 days as described previously [22–24]. These treatments have been shown to induce mild metabolic acidosis or alkalosis in rodents [24, 25]. The control group received only 2% sucrose in their drinking water. An additional group of mice were placed on a K^+-depleted diet for 7 days (Bio-serv, Frenchtown, NJ, USA) as described previously [26, 27]. Each group consisted of seven animals for each time point and treatment, respectively. Three animals of each group were used for immunohistochemistry and the remaining four for Western blotting. All studies were approved by the Yale Animal Care and Use Committee.

Western blotting

Mice were sacrificed and the kidneys rapidly harvested. Microdissected cortex and medulla or total kidneys were homogenized in an ice-cold K-HEPES buffer (200 mmol/L mannitol, 80 mmol/L K-HEPES, 41 mmol/L KOH, pH 7.5) with pepstatin, leupeptin, K-ethylenediaminetetraacetic acid (K-EDTA), and phenylmethylsulfonyl fluoride (PMSF) added as protease inhibitors. The samples were centrifuged at 1000 $\times$ *g* for 10 minutes at 4°C and the supernatant saved. Subsequently, the supernatant was centrifuged at 100,000 $\times$ *g* for one hour at 4°C and the resultant pellet resuspended in K-HEPES buffer containing protease inhibitors. After measurement of the total protein concentration (Biorad Protein kit; Bio-Rad, Richmond, CA, USA), 50 μg of crude membrane protein were solubilized in Laemmli sample buffer, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% polyacrylamide gels. For immunoblotting, proteins were transferred electrophoretically from unstained gels to polyvinylidine difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA, USA). After blocking with 5% milk powder in Tris-buffered saline/0.1% Tween-20 for 60 minutes, the blots were incubated with the primary antibodies (rabbit anti-pendrin raw serum 1:10,000 [2] and mouse monoclonal anti-actin (42 kD; Sigma Chemical Co., St. Louis, MO, USA) 1:500 either for two hours at room temperature or overnight at 4°C. After washing and subsequent blocking, blots were incubated with the secondary antibodies (donkey anti-rabbit 1:10,000 and sheep anti-mouse 1:5000) IgG-conjugated with horseradish peroxidase (Amersham Life Sciences, Braunschweig, Germany) for one hour at room temperature. Antibody binding was detected with the enhanced chemiluminescence ECL kit (Amersham Pharmacia Biotech) before exposure to X-ray film (Eastman Kodak, Rochester, NY, USA). All films were analyzed using SCION Imaging software. Two bands of about 110 and 220 kD were detected for pendrin as shown in Figure 1. Labeling of both bands was blocked by preincubation of the anti-pendrin antibody with the

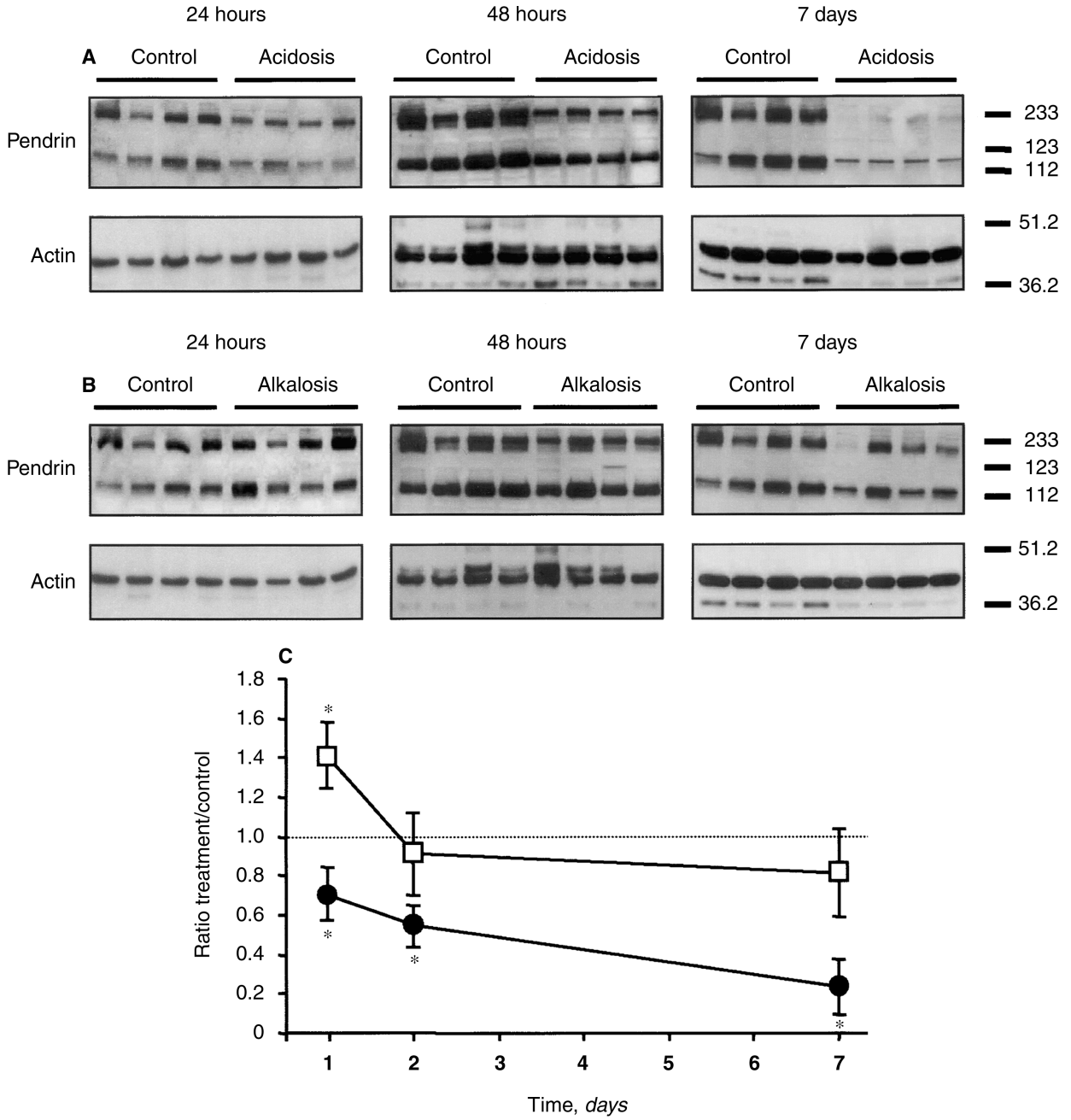

Fig. 2. Expression of pendrin in total mouse kidney membrane fractions in response to NH_4Cl (0.28 mol/L) or $NaHCO_3$ (0.28 mol/L) loading for 24 and 48 hours and 7 days. For the calculation of the pendrin/actin ratio, both pendrin specific bands were pooled. (*A*, *B*) Pendrin expression in response to NH_4Cl and $NaHCO_3$ loading in 4 mice. Membranes were stripped and reprobed with an antibody against the 42 kD actin subunit to control for protein-loading. (*C*) Ratio (treated:control) of pendrin:actin in kidneys from control and treated mice. In NH_4Cl-loaded animals (●) pendrin expression was significantly reduced after 48 hours and 7 days. In $NaHCO_3$-loaded mice (□) pendrin expression was significantly elevated after 24 hours and returned to normal levels at 48 hours and 7 days. All samples were used for three independent Western blots and the ratio of pendrin:actin was calculated for each experiment as described in the **Methods** section. Data represent mean ± SEM. Asteriks (*) mark significantly increased or decreased protein levels in treatment group compared to controls.

immunizing peptide, and both bands responded similarly to the different treatments. The signals of both bands were pooled for the subsequent calculation of pendrin protein levels. For each time point and sample, the ratio of pendrin:actin was determined and used to calculate the ratio between the control group and treated group using Gauss' law of error propagation. All results were tested for significance using the unpaired Student *t* test and only results with $P < 0.05$ were considered as statistically significant.

Immunohistochemistry

Mice were anesthetized with pentobarbital and perfused through the left ventricle with phosphate-buffered saline (PBS) followed by paraformaldehyde-lysine-periodate (PLP) fixative [28]. Kidneys were removed and fixed overnight at 4°C by immersion in PLP. Kidneys were washed three times with PBS and 5 μm cryosections were cut after cryoprotection with 2.3 mol/L sucrose in PBS for at least 12 hours. Immunostaining was carried out as described previously [29]. Sections were incubated with 1% SDS for five minutes, washed three times with PBS and incubated with PBS containing 1% bovine serum albumin (BSA) for 15 minutes prior to the primary antibody. The primary antibodies [rabbit anti-pendrin raw serum 1:300 and goat anti-human aquaporin-2 (AQP-2; Santa Cruz Technology, Santa Cruz, CA, USA) 1:100] were both diluted in PBS and applied either for 75 minutes at room temperature or overnight at 4°C. Sections were then washed twice for five minutes with high NaCl PBS (PBS + 2.7% NaCl), once with PBS, and incubated with the secondary antibodies (donkey anti-rabbit Cy3 and donkey anti-goat Cy5 at dilutions of 1:1000 and 1:100, respectively; Jackson ImmunoResearch) for one hour at room temperature. Sections were again washed twice with high NaCl PBS and once with PBS before mounting with VectaMount (Vector Laboratories, Burlingame, CA, USA). Sections were viewed with a Zeiss LSM 410 confocal microscope (Zeiss, Jena, Germany). For cell counts, 10 cortical fields from each sample at ×400 magnification were imaged and cells counted as either positive for AQP-2 or negative for AQP-2 and positive or negative for pendrin staining.

RESULTS

A previously described antibody raised against the C-terminal region of rat pendrin [2] was used to test for pendrin expression in crude membranes prepared from mouse renal cortex. As shown in Figure 1, a 110 kD band was detected, corresponding to the expected molecular mass of monomeric pendrin; in addition, a 220 kD band was detected, likely representing a pendrin dimer. Labeling of both bands was blocked when the antibody was preincubated with the immunizing peptide, indicating antibody specificity (Fig. 1). Neither band was detected in membranes prepared from mouse renal medulla. However, a weak 60 kD band was seen in medulla that could also be protected by preincubation with the immunizing peptide. The identity of the recognized protein is unknown and may represent a related protein.

Using total kidney crude membrane preparations, Western blotting showed that pendrin expression was significantly reduced after 24 and 48 hours of acid loading and had decreased to 23.2 ± 14.1% of control levels by day 7 (Fig. 2 A, C). In contrast, in kidneys from $NaHCO_3$-treated animals pendrin expression increased slightly after 24 hours but returned to normal levels at the later time points (Fig. 2 B, C).

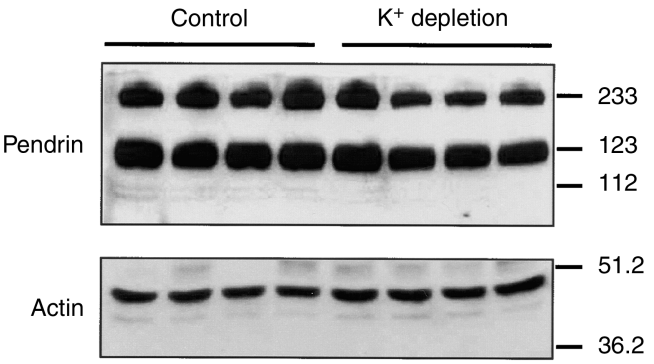

Fig. 3. Effect of K^+-depletion on pendrin expression. Chronic K^+-depletion for 7 days reduced pendrin expression by 30%. The ratio of pendrin:actin in K^+-depleted mice was only 70.2 ± 12.9% compared to animals on normal diet.

Chronic potassium depletion induces metabolic alkalosis through a variety of mechanisms including shifting extracellular protons to the intracellular space in exchange for K^+, and increasing activity or expression of bicarbonate reabsorbing mechanisms in the kidney [30–33]. In the K^+-depleted animals pendrin expression was reduced to 70.2 ± 12.9% of control levels observed in animals on normal diet (Fig. 3).

Immunohistochemistry confirmed that expression of pendrin was limited to the cortical distal segments of the nephron (Figs. 4A and 5A). Staining was absent from the medullary collecting duct (Fig. 4B). Preincubation of the antibody with the immunizing peptide abolished all staining whereas AQP-2 staining was not affected (Fig. 4 C, D). Expression of pendrin was found only on the apical pole in a subset of cells negative for the principal cell specific water channel AQP-2 (Fig. 4A). This is consistent with the recent localization of pendrin in non-type A intercalated cells in mouse, rat, and human kidney [8].

After 48 hours of acid loading, pendrin was partially redistributed to the cytosol but also was seen in the apical membrane (Fig. 5 B, C). In addition, after seven days of acidosis, the number of cells that were both AQP-2-negative and pendrin positive was greatly reduced with the remaining pendrin staining very weak (Figs. 5D and 6). Similar to the findings in kidneys from acid-loaded mice, chronic K^+-depletion for one week also caused a redistribution of pendrin protein to the cytosol whereas the relative abundance of pendrin positive and AQP-2 negative cells changed only slightly (Figs. 5F and 6).

In contrast, after 48 hours and 7 days of alkali loading (0.28 mol/L $NaHCO_3$), pendrin expression was confined to a small apical rim and the number of cells that were both AQP-2 negative and pendrin positive was increased

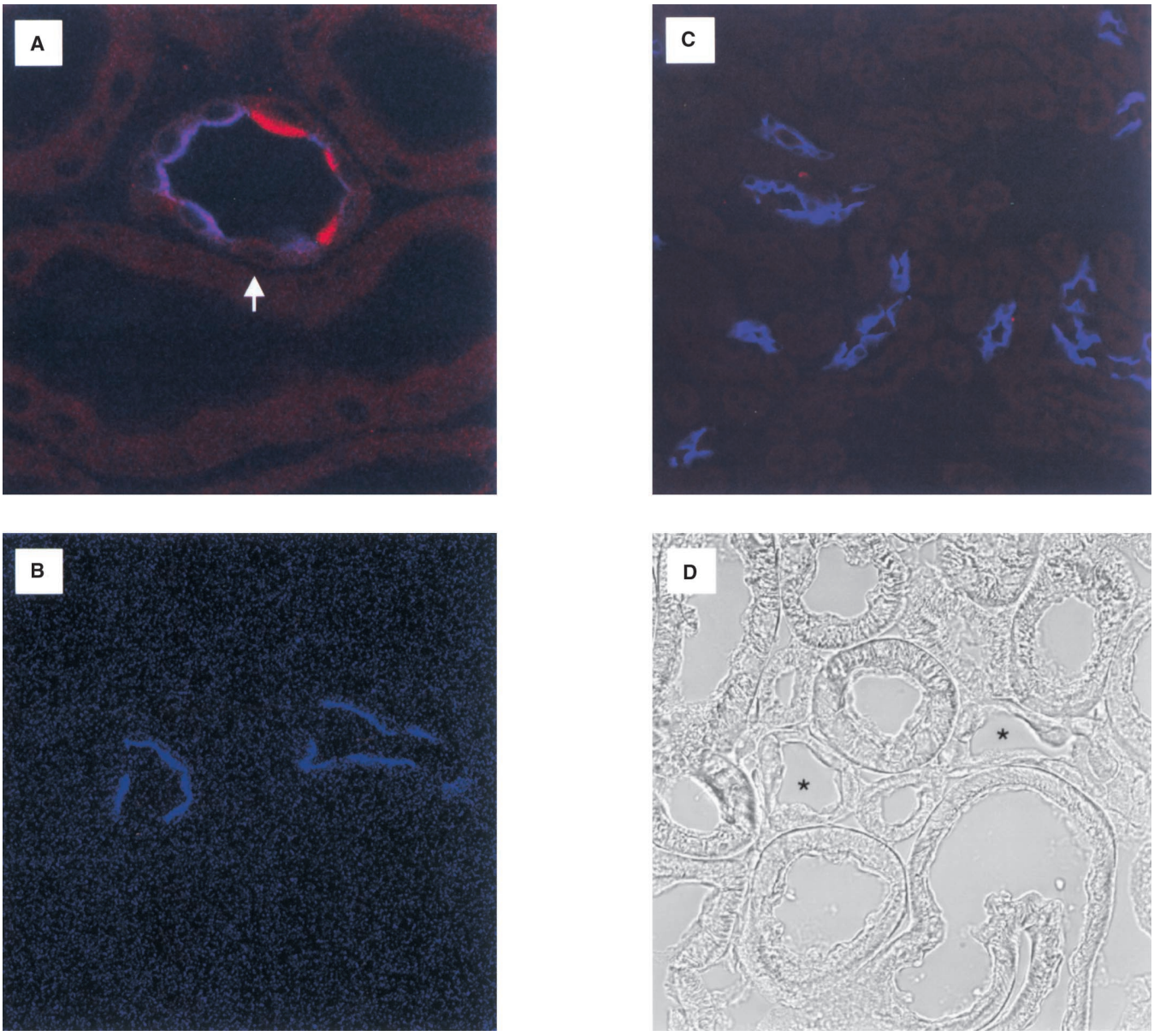

Fig. 4. Expression of pendrin in mouse kidney by immunohistochemistry. (*A*) Expression of pendrin (red) in the cortical collecting duct. Sections were costained for the principal cell specific water channel aquaporin 2 (AQP-2, blue). Not all AQP-2 negative cells were positive for pendrin staining (arrow) (original magnification ×800). (*B*) Pendrin expression was not detected in the medulla (original magnification ×400). (*C*) Preincubation of the anti-pendrin antibody with the immunizing peptide eliminated pendrin staining without altering staining of AQP-2. (*D*) Phase contrast picture of the same area as shown in C (original magnification ×800).

(Figs. 5E and 6). The number of AQP-2 and pendrin negative cells, however, was significantly reduced compared to control conditions.

DISCUSSION

Pendrin belongs to a recently emerging superfamily of Cl^-/anion exchangers [1–6]. Some of these transporters, such as DRA (down-regulated in adenoma), are involved in the transcellular transport of bicarbonate or may be linked to the regulation of intracellular pH. Indeed, it has been demonstrated that pendrin transports bicarbonate in addition to other anions such as iodide or formate [12, 13, 34]. Recently, Royaux et al demonstrated reduced bicarbonate secretion in cortical collecting ducts from alkalotic pendrin knockout (pds −/−) mice, suggesting that pendrin may be involved in apical bicarbonate secretion by B-type intercalated cells [8]. Accordingly, pendrin expression was found in a subset of intercalated cells in human kidney that were negative for the type A cell

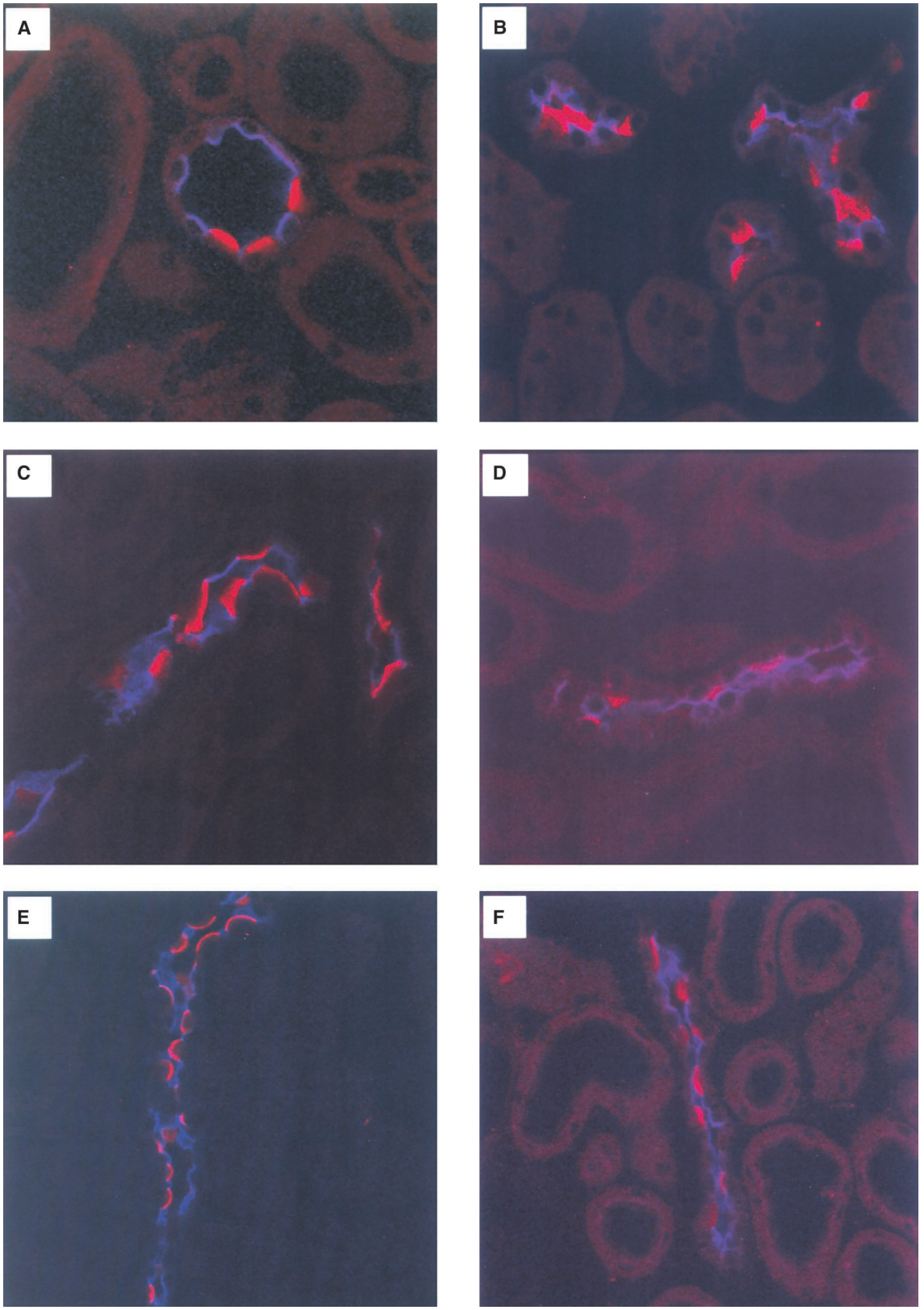

Fig. 5. Effects of NH_4Cl and $NaHCO_3$ loading, and chronic K^+-depletion on pendrin localization and on the abundance of pendrin positive cells in the cortical collecting duct in mouse kidney. (*A*) Under control conditions, pendrin is primarily found in the apical compartment and the majority of AQP-2 negative cells are positive for pendrin expression (original magnification ×560). (*B*, *C*) After 48 hours of NH_4Cl-loading a marked redistribution of pendrin expression is seen, with a more cytosolic localization (original magnifications ×800, ×560). (*D*) After 7 days of NH_4Cl-loading pendrin is found mainly in the cytosol. There is a striking reduction in AQP-2 negative and pendrin positive cells (original magnification ×800). (*E*) $NaHCO_3$-loading for 48 hours led to the pronounced apical localization of pendrin in the cortical collecting duct (original magnification ×400). (*F*) Chronic K^+-depletion for 1 week caused a redistribution of pendrin with more cytosolic localization similar to 48 hours of NH_4Cl-loading (original magnification ×800).

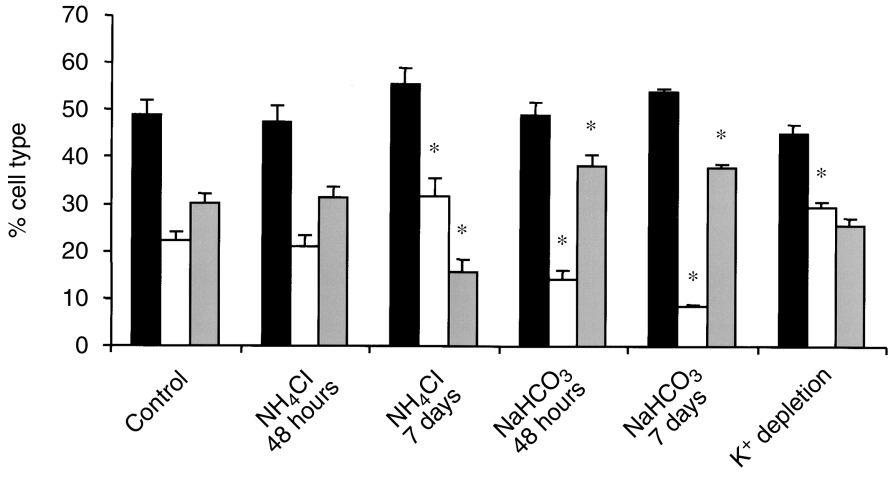

Fig. 6. Relative abundance of three cortical collecting duct cell types defined by their expression patterns of AQP-2 and pendrin. AQP-2 is a marker for principal cells in the collecting duct and AQP-2 positive cells, thus, represent principal cells. Intercalated cells are AQP-2 negative and may be divided into two subtypes (pendrin + and pendrin −). Symbols are: (■) AQP-2 +/pendrin −; (□) AQP-2 −/pendrin −; (▩) AQP-2 −/pendrin +. Royaux et al suggested that pendrin positive cells may reflect non-type A cells as they fail to express the type A cell specific kAE-1 protein [8]. After 7 days but not 48 hours of NH_4Cl loading the relative number of pendrin positive/AQP-2 negative cells decreased (from 30.2 ± 1.8% to 15.7 ± 2.7%), whereas the number of cells negative for both pendrin and AQP-2 increased (from 22.2 ± 2.2% to 31.8 ± 3.6%), consistent with an increase in the number of acid secreting type A intercalated cells. In contrast, after 48 hours and 7 days of $NaHCO_3$-loading the percentage of pendrin positive/AQP-2 negative cells increased (from 30.2 ± 1.8% to 37.8 ± 1.0%) and the number of pendrin negative/AQP-2 negative cells was reduced (from 22.2 ± 2.2% to 8.4 ± 0.5%) suggesting an increase in bicarbonate secreting type B intercalated cells. Mild K^+-depletion for 1 week led to an increase in the relative number of pendrin negative/AQP-2 cells to 29.3 ± 1.4%. Data represent the mean ± SEM from ten fields from two sections of three animals for each condition. The data for each animal were pooled before calculating the average. Significant changes in the relative abundance (*) of aquaporin 2 negative cells (AQP-2 −) and pendrin positive (+) or pendrin negative (−) cells were found in response to NH_4Cl- and $NaHCO_3$-loading, and chronic K^+-depletion.

marker AE1. This subset thus may represent both B cells and potentially the third (non-A/non-B or gamma) subtype of intercalated cells described by some authors [19–21]. Functionally, B cells are responsible for bicarbonate secretion that is activated in metabolic alkalosis and inactivated in metabolic acidosis [14, 15, 35, 36]. The mechanisms by which activation and inactivation occur are not fully understood at present. The expression of pendrin in non-type A intercalated cells and the reduced ability of cortical collecting ducts from pendrin deficient mice to secrete bicarbonate in response to metabolic alkalosis strongly suggests an involvement of pendrin in bicarbonate secretion by B cells.

The present study examined the regulation of renal pendrin expression in mice treated with NH_4Cl, or $NaHCO_3$-loading or with chronic K^+-depletion. Expression of pendrin was strongly affected by metabolic acidosis in three ways: (*1*) pendrin protein levels decreased significantly, (*2*) pendrin had a more pronounced cytosolic expression pattern, and (*3*) the relative abundance of cells both positive for pendrin and negative for AQP-2 was reduced.

In contrast to the changes seen in NH_4Cl-treated or K^+-depleted animals, $NaHCO_3$-loading caused a transient increase in pendrin expression at an early time point that later returned to normal levels. Associated with these changes was a pronounced redistribution of pendrin to the apical membrane. The relative abundance of pendrin positive cells also was increased.

Chronic K^+-depletion leads to the development of metabolic alkalosis through a variety of mechanisms [30]. On a cellular level, exchange of intracellular K^+ with extracellular H^+ occurs. In the kidney, bicarbonate and potassium-saving as well as H^+-secreting mechanisms such as the Na^+/HCO_3^- cotransporter kNBC1, the colonic H^+/K^+-ATPase, and the vacuolar H^+-ATPase are stimulated or up-regulated [31–33, 37]. Morphologically, hyperplasia of intercalated cells has been described [38]. The facts that metabolic alkalosis did not change pendrin protein expression levels after seven days of HCO_3^--loading and that K^+-depletion led to a reduction of pendrin protein levels suggests that the K^+-depletion itself—but not the resulting metabolic alkalosis—is responsible for the reduction in pendrin expression levels. Thus, the reduction

in pendrin protein levels, the redistribution of pendrin to the cytosol, and the reduction in the relative abundance of pendrin positive cells seen in response to K^+-depletion may contribute to and support the development of metabolic alkalosis seen in the setting of K^+-depletion.

The present findings demonstrate that pendrin expression in mouse kidney is regulated by dietary acid-base and electrolyte intake. Three different responses have been identified: (*1*) changes in protein levels; (*2*) differences in subcellular location of pendrin, possibly reflecting trafficking of pendrin; and (*3*) changes in the relative abundance of pendrin positive cells. Activation of bicarbonate secreting cells has been demonstrated in response to metabolic alkalosis and both the activation of resting cells as well as an increase in the relative abundance of this cell-type postulated [14, 15, 35, 36, 39]. Thus, increases in both pendrin expression and trafficking to the apical membrane may provide part of the underlying mechanism of activation of resting B cells. Based on the substrates identified to be transported by pendrin (that is, iodide, formate, bicarbonate) it needs to be further clarified if pendrin itself secretes bicarbonate or is involved in recycling other substrates required for B-cell–mediated bicarbonate secretion. Considerable concentrations of formate are found in final urine, but so far there is no evidence that formate is involved in transport processes in the connecting segment or collecting duct. However, it has been shown to stimulate Na^+ and Cl^- reabsorption in the early but not late distal convoluted tubule through activation of Na^+/H^+-exchange in parallel with Cl^-/organic anion exchange [40].

The last finding, the increase in the relative abundance of pendrin positive cells, might reflect intercalated cell plasticity, such as the conversion of type A to type B cells and vice versa. The cortical collecting duct adapts to changes in acid-base status by increasing or decreasing the number of type A or B intercalated cells while keeping the overall number of intercalated cells constant. This interconversion of intercalated cells has been described functionally in isolated perfused cortical collecting ducts from rabbit [35, 36, 39, 41, 42] as well as in primary cell cultures [43–45] and may require the production, secretion, and presence of the extracellular matrix protein hensin [14, 42, 46]. However, it remains to be clarified in vivo if interconversion or changes in cell proliferation and removal occur. The underlying signaling pathways as well as molecular events have remained elusive to date. The fact that the relative portion of pendrin positive cells was altered in response to acid-base and electrolyte intake may help in devising a strategy to investigate cortical collecting duct cell plasticity in future studies.

In summary, our results provide evidence for renal pendrin expression regulation in response to acid-base and electrolyte intake. Pendrin expression appears to be regulated on three levels: pendrin protein levels, pendrin trafficking, and intercalated cell conversion. Therefore, these results support the notion that pendrin may be involved in acid-base handling by the cortical collecting duct and may play a role in bicarbonate or base secretion by B cells. Interestingly, chronic K^+-depletion reduced pendrin expression, suggesting a loss of the bicarbonate or base secretory ability of B cells under this condition. However, whether pendrin itself secretes bicarbonate or rather recycles other ions necessary for bicarbonate secretion will need further clarification.

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