Adaptive redistribution of NBCe1-A and NBCe1-B in rat kidney proximal tubule and striated ducts of salivary glands during acid-base disturbances

Alena Brandes, Oliver Oehlke, Anne Schümman, Stefanie Heidrich, Frank Thévenod, and Eleni Roussa

1Center for Anatomy, Department of Neuroanatomy, Georg-August-University Goettingen, Goettingen, Germany; and 2Institute for Physiology and Pathophysiology, University of Witten-Herdecke, Witten, Germany

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Brandes A, Oehlke O, Schümman A, Heidrich S, Thévenod F, Roussa E. Adaptive redistribution of NBCe1-A and NBCe1-B in rat kidney proximal tubule and striated ducts of salivary glands during acid-base disturbances. Am J Physiol Regul Integr Comp Physiol 293: R2400–R2411, 2007. First published September 12, 2007; doi:10.1152/ajpregu.00208.2007—The cellular distribution of the NH2-terminal electrogenic Na\(^{+}\)-HCO\(_3\)\(^{-}\) cotransporter (NBCe1) variants NBCe1-A and NBCe1-B has been investigated in rat kidney and submandibular gland (SMG) under physiological conditions and after systemic acid-base perturbations. Moreover, the in vivo data were complemented in vitro by using an immortalized cell line derived from the S1 segment of the proximal tubule (PT) of normotensive Wistar-Kyoto rats (WKPT-0293 C1.2). NBCe1-A was basolaterally localized in PT cells, whereas NBCe1-B exhibited intracellular and basolateral distribution. SMG showed transcript and protein expression for NBCe1-A and NBCe1-B. NBCe1-B was basolaterally localized in duct cells; NBCe1-A was found intracellularly in salivary striated ducts and apically in main duct cells. Acute metabolic acidosis significantly increased cells that showed basolateral NBCe1-A in the PT, indicating increased HCO\(_3\)\(^{-}\) reabsorption, and significantly decreased cells that exhibited basolateral NBCe1-B in the salivary ducts, suggesting decreased HCO\(_3\)\(^{-}\) secretion. Chronic acidosis had no effect on NBCe1 distribution in PT but significantly increased the percentage of cells with basolateral NBCe1-A in salivary striated duct cells, suggesting increased HCO\(_3\)\(^{-}\) reabsorption. In contrast, chronic alkalosis caused adaptive redistribution of NBCe1-A and NBCe1-B in renal PT, favoring decreased HCO\(_3\)\(^{-}\) reabsorption. In vitro, WKPT-0293 C1.2 cells expressed key acid-base transporters. Extracellular alkalosis downregulated NBCe1-A protein. WKPT-0293 C1.2 cells are therefore a useful model to study renal acid-base regulation in vitro. The results propose redistribution of the transporters as a potential posttranslational regulation modus during acid-base disturbances. Moreover, the data demonstrate that renal PT and salivary duct epithelia respond to acid-base disturbances by an opposite redistribution pattern for NBCe1-A and NBCe1-B, reflecting specialized functions as the HCO\(_3\)\(^{-}\)-reabsorbing and HCO\(_3\)\(^{-}\)-secreting epithelium, respectively.

acid-base homeostasis; bicarbonate transport

ORIGINALLY, TWO MEMBERS OF the SLC4A4 gene family, the NH2-terminal variants of the electrogenic Na\(^{+}\)-HCO\(_3\)\(^{-}\) cotransporter (NBC1), were identified and designated as kidney (kNBC1) and pancreas “specific” (pNBC1) (2, 27, 28). They are now termed NBCe1-A and NBCe1-B, respectively, but are by no means specific for the organs their previous names originated from. Both variants have been detected in pancreas, kidney, intestine, and eye (8, 13, 30, 34, 36).

NBCe1 distribution, regulation, and physiological significance in the kidney have been extensively studied. NBCe1-A is exclusively localized in the basolateral membrane of proximal tubule (PT) cells (7, 26), operates with a stoichiometry of 1 Na\(^{+}\):3 HCO\(_3\)\(^{-}\), and is involved in HCO\(_3\)\(^{-}\) reabsorption in the proximal nephron. Several mutations of the SLC4A4 gene have been reported that led to characteristic phenotypes, including proximal renal tubular acidosis and ocular abnormalities (12, 16–18). Among this line, NBCe1 knockout mice exhibit severe metabolic acidosis (14). Conversely, changes of systemic acid-base balance apparently influence Na\(^{+}\)-HCO\(_3\)\(^{-}\) cotransporter activity. According to functional studies, the Na\(^{+}\)-HCO\(_3\)\(^{-}\) cotransport activity is increased during metabolic acidosis and decreased during metabolic alkalosis (3, 25, 37). At the molecular level, however, these observations have not yet been confirmed. During metabolic acidosis, NBCe1 mRNA (7) and protein levels (5, 13, 19) remained unchanged compared with the controls, suggesting that other posttranslational regulatory mechanisms may be operative. In addition, NBCe1-B is also expressed in the renal PT, and showed a diffuse intracellular distribution (30), the functional role of which is not clear.

Whereas the PT is a prototype for a HCO\(_3\)\(^{-}\)-reabsorbing epithelium, the salivary ducts are specialized to secrete HCO\(_3\)\(^{-}\). Salivary duct cells are equipped with several transport proteins that reveal a polarized distribution and therefore enable trans epithelial ion fluxes (11). Although the apical Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanger of duct cells mediating HCO\(_3\)\(^{-}\) secretion remains unidentified at the molecular level, recent functional and immunohistochemical studies have demonstrated that NBCe1 is involved in the mechanisms of HCO\(_3\)\(^{-}\) transport in salivary glands and is therefore a potential modulator of the final HCO\(_3\)\(^{-}\) concentration of the saliva (21, 29, 31). Interestingly, in the NBCe1-deficient mouse, the physiological relevance of the transporter in the gastrointestinal tract has been revealed (14). In NBCe1 knockout mice, the HCO\(_3\)\(^{-}\) uptake during cAMP-stimulated anion secretion in the proximal colon is impaired.

We (29, 31) previously characterized cellular distribution and subcellular localization of NBCe1 in rat and human salivary glands by using an antibody raised against a COOH-terminal sequence of NBCe1, which therefore does not discriminate between the NH2-terminal variants. In the rat submandibular gland (SMG), NBCe1 was basolaterally distributed in striated duct cells and lumina located in the main excretory duct cells. These data have been recently confirmed at the...
functional level in duct cells of guinea pig salivary glands (21). However, the cellular distribution of both NBCe1 variants in salivary glands has not been investigated so far.

Taking these data into account, along with the observation that acid-base changes influence localization of $H^+$ and $HCO_3^-$ transporters not only in the kidney but also in salivary duct epithelium (24, 32), we investigated mRNA and protein expression as well as cellular distribution of NBCe1-A and NBCe1-B in the rat kidney and SMG under physiological conditions and following acute and chronic metabolic acidosis and alkalosis in vivo. Moreover, the in vivo kidney data were complemented by in vitro experiments using a rat kidney PT cell line.

MATERIALS AND METHODS

Antibodies. Generation of anti-peptide antibodies against variant specific regions in the NH$_2$ terminus of NBCe1 has been described earlier (30). The ε332 antibody is directed against amino acids 60–77 of rat NBCe1-B (ISENVYSDKSIENADESS), specific for the “mede” start (40). The ε333 antibody is directed against amino acids 1–23 of rat NBCe1-A (MSTENVEGPKPNLGERGRARSST), specific for the short “mste” start (28). Generation of the antibodies against the ε-subunit of vacuolar (V)-ATPase has been previously described (32). The mouse monoclonal antibody against Na$^+$/H$^+$ exchanger 3 (NHE3) was purchased from Chemicon. The antibody against GAPDH was from Abcam (Cambridge, UK). The mouse monoclonal anti-Na$^+$/K$^+$-ATPase antibody was from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Goat anti-rabbit or anti-mouse IgG coupled to horseradish peroxidase (HRP; DAKO, Hamburg, Germany), FITC, and Cy3 (Jackson Immunoresearch, Suffolk, UK) were used as secondary antibodies.

Induction of acute and chronic metabolic acidosis and alkalosis. Procedures were approved by the Animal Ethics Committee, and animal handling was in accordance with the German Law on Animal Experimentation and the European Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (86/609/EU). Male Wistar rats (200–300 g) were used. The six experiments were as follows: acute control, acute acidosis, acute alkalosis, chronic control, chronic acidosis, and chronic alkalosis. Each group consisted of six to seven animals, and experiments were repeated at least three times. Acute metabolic acidosis and alkalosis were induced by gastric administration of 1.5 M NH$_4$Cl (2.4 ml/100 g body wt) or of 0.976 M NaHCO$_3$ (5 ml/100 g body wt), respectively (33). Control animals were treated with 1.37 M NaCl (2.4 ml/100 g body wt) (33). The animals were killed 6 h later. Chronic acidosis and alkalosis were induced according to Wright and Knepper (43), with additional administration of 0.28 M NH$_4$Cl or 0.28 M NaHCO$_3$, respectively, in the drinking water for 6–9 days. Control animals received 0.28 M NaCl in their drinking water.

The animals were anesthetized with pentobarbital sodium (Nembutal, 65 mg/kg ip). After collection of arterial blood from the abdominal aorta and urine from the urinary bladder, pH, PCO$_2$, HCO$_3^-$, percent O$_2$ saturation were determined in blood and urine (Table 1). Subsequently, the animals were perfusion-fixed with 2% paraformaldehyde–75 mM lysine–10 mM sodium periodate (PLP) (23), and SMGs were excised.

RT-PCR. Rats were killed by cervical dislocation, and SMGs were quickly excised and immediately frozen in liquid N$_2$. Total RNA was isolated from rat SMG tissue by using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Total RNA (1.0 μg) was reverse transcribed from an oligo(dt) primer by using a Qiagen Omniscript kit. Five microliters of the RT reaction were used in a PCR containing 2.5 U Tag DNA polymerase, 0.2 μM specific primers, 1× Qiagen PCR buffer, 200 μM deoxynucleoside triphosphates, and 2 mM MgCl$_2$. The following transporterspecific primers were used: NBCe1-A, forward primer 5′-AGTTTGCGCCACCCACCAAC-3′ and reverse primer 5′-CCCTGTTCCCCACTTTCTTCT-3′ (the amplified product corresponds to nt 4–347; GenBank accession no. AF027362; Ref. 7); NBCe1-B, forward primer 5′-TCATCTCGGTCGGCCAAA-3′ and reverse primer 5′-CCCTGGTTCACCTTCTCTCACA-3′ (the amplified product corresponds to nt 10-482; GenBank accession no. AF210250; Ref. 15); NHE3, forward primer 5′-CAAGGCACCTAGGTTTATAGCATACG-3′ and reverse primer 5′-TCTCTTCCTCAGATAAGGTGTCGAACACT-3′ (the amplified product corresponds to nt 3011–3209; GenBank accession no. NM_012654.1); V-ATPase (ε-subunit), forward primer 5′-CCTGGCCCGACCTTTG-3′ (nt 85–101) and reverse primer 5′-CCATCATGTGCTTAATCTGCTTCT-3′ (nt 157–134; GenBank accession no NM_198745). For amplification of the housekeeping gene actin, the following primers were used: forward primer 5′-TTAAAAGGACCTCCTAGAATACGCCCCTCCACACT-3′ and reverse primer 5′-TGGAATCCTGTGGCATCCATGAAC-3′. Sensitivity and specificity of the primers were confirmed by sequencing of the PCR products. The conditions used for PCR amplification were as follows: 30 s at 95°C, 30 s at 55°C, and 60 s at 72°C; 35 cycles were performed. The amplicons were separated on 2% agarose gels, stained with ethidium bromide.

Preparation of tissue homogenate, isolation of plasma membranes, and immunoblotting. Male Wistar rats (n = 4–6) were anesthetized with ether and were perfused via the left ventricle with warm phosphate-buffered saline (PBS; 37°C) until the organs were thoroughly blanched. SMG homogenate and plasma-membrane fractions were prepared as previously reported (38, 40). SMG homogenate was prepared by grinding the respective tissue with 50 strokes of a motor-driven Potter homogenizer in 10 ml of ice-cold homogenizing buffer containing (in mM) 280 mannitol, 10 HEPES, 10 KCl, 1 MgCl$_2$ adjusted to pH 7.0, and a protease inhibitor cocktail (10 μM leupeptin, 2 mM benzamidine, and 0.1 mM Pefabloc SC). The homogenate was centrifuged at 50,000 g for 5 min. To obtain plasma membrane-enriched fractions, the cleared homogenate was centrifuged for 12 min at 1,000 g and the supernatant was centrifuged at 11,000 g for 15 min. The 11,000-g pellet was composed of a whitish fluffy upper layer and a yellowish bottom layer, which were separated. The 11,000-g fluffy layer was mixed with 2.0 M sucrose buffer to a concentration of 1.25 M, which was layered on 2.0 M sucrose and overlaid with 0.3 M sucrose. The gradient was centrifuged at 140,000 g for 90 min, and the whitish band enriched in plasma membranes at the upper surface between the 0.3 M and 1.25 M sucrose-density layers was collected.

Table 1. Acid-base parameters

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<th>Acute Acidosis</th>
<th>Control</th>
<th>Acute Alkalosis</th>
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<tr>
<td></td>
<td>n = 7</td>
<td>n = 7</td>
<td>n = 7</td>
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<tr>
<td>Blood ph</td>
<td>7.14 ± 0.07</td>
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<td>7.43 ± 0.03</td>
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<td>PCO$_2$</td>
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<td>HCO$_3^-$, mM</td>
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<td>21.56 ± 1.74</td>
<td>30.78 ± 1.15</td>
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<tr>
<td>Urine pH</td>
<td>5.43 ± 0.11</td>
<td>6.18 ± 0.39</td>
<td>7.07</td>
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Values are means ± SE; n = number of animals.

Cell monolayers from an immortalized cell line derived from the S1 segment of the PT of normotensive Wistar-Kyoto rats (WKPT-0293 C1.L2) were washed three times with homogenization buffer, scraped off the culture flasks with a rubber policeman, pelleted by centrifugation at 250 g for 5 min, and resuspended in homogenization buffer.
buffer. Homogenization was performed by sonication. Protein concentration was determined according to Bradford (10), and samples were processed for immunoblotting. Electrophoresis and blotting procedures were performed as previously described (24, 30). Proteins were separated by SDS-PAGE and were transferred to PVDF membranes. Blots were blocked with 3% non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20 and were incubated with primary antibody overnight at a dilution of 1:1,000 for NBCe1-A and NBCe1-B and a dilution of 1:10,000 for GAPDH. After incubation with goat anti-rabbit or anti-mouse IgG coupled to HRP secondary antibodies (1:10,000 dilution), blots were developed in enhanced chemiluminescence reagents and signals were visualized on X-ray film. Subsequently, films were scanned by using a flat-bed scanner, and the signal ratio of NBCe1-A or NBCe1-B to GAPDH was quantified densitometrically (optical density). Differences in signal ratio were tested for significance by using Student’s t-test. Results with levels of \( p < 0.05 \) were considered significant.

**Immunohistochemistry.** PLP-fixed rat SMG and kidney specimens were cut into small blocks, cryoprotected in 30% sucrose, and frozen in liquid N2. Indirect immunohistochemistry was performed on 5-μm cryosections (31). Sections were rehydrated in PBS, treated with 1% SDS for 5 min, blocked with 1% bovine serum albumin/PBS, and incubated with primary antibody (anti-NBCe1-A or anti-NBCe1-B) overnight at 4°C at a dilution of 1:100. Slides were washed and were incubated with goat anti-rabbit IgG coupled to HRP (1:50 dilution) for 1 h at room temperature, and peroxidase activity was visualized with 3,3′-diaminobenzidine and hydrogen peroxide. Sections were dehydrated in a graded series of ethanol, cleared in xylene, and mounted with Entellan (Merck, Darmstadt, Germany). Labeled sections were viewed with a light microscope (Zeiss), and images were recorded with an Axioscam digital camera connected to an IBM-compatible personal computer. For cell counting, only cells with a distinct nucleus were considered. All sections were examined, and cell counting was performed in single-blind experiments by two independent experimenters. Immunoreactivity was classified into absent, intracellular, apical, or basolateral. At least 150 striated duct cells or renal PT cells were counted in one representative tissue section per animal and were processed for immunoblotting.

**In situ hybridization.** For in situ hybridization, cells were fixed in 4% paraformaldehyde for 30 min at room temperature, treated with 1% SDS for 15 min, and incubated overnight at 4°C with primary antibodies diluted in PBS. The following day cells were incubated with FITC- or Cy3-conjugated donkey anti-rabbit or mouse IgG secondary antibodies for 1 h at room temperature. Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) diluted 1:1,000 in PBS for 5 min, washed with PBS, mounted with Vectashield, and viewed with a Zeiss Axioskop fluorescence microscope.

**Electron microscopy.** Confluent monolayers of WKPT-0293 C1.2 cells were fixed in situ for 2 h with 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate, pH 7.4. The cell monolayer was dehydrated and embedded in Spurr’s resin. Ultrathin sections were stained with uranyl acetate and lead citrate before analysis of the sections with a Zeiss Leo 906E electron microscope.

**RESULTS**

**NBCe1-A and NBCe1-B distribution in the rat PT under physiological conditions and after systemic acid-base disturbances.** NBCe1 is expressed in the renal PT, where it contributes to HCO3− reabsorption in this nephron segment. In addition, it is well established that the kidney responds to acid-base disturbances by adaptive processes in the proximal and distal nephron (4, 6, 9). We therefore investigated whether acute or chronic metabolic acidosis and alkalosis affect distribution of NBCe1-A and NBCe1-B in renal PT.

The effects of acute and chronic metabolic acidosis and alkalosis on NBCe1-A expression and distribution in rat renal PT are illustrated in a representative experiment shown in Fig. 1, where double immunofluorescence with antibodies against NBCe1-A (red) and NHE3 (green) an apical membrane mark-
ers was performed. Similar results were obtained by immunohistochemistry using HRP-coupled secondary antibodies (data not shown). Quantitative analysis of immunohistochemical sections revealed basolateral NBCe1-A immunoreactivity in 87 ± 0.9% of control PT cells, and 13 ± 0.9% exhibited intracellular NBCe1-A localization (Fig. 1G), confirming previous results (30). During acute metabolic acidosis, the number of basolaterally stained cells was found modestly, but nevertheless significantly, increased (92.4 ± 1.1%; \(P < 0.05\); Fig. 1, B and G) compared with the controls. Consequently, acute metabolic acidosis also resulted in a significant decrease in the number of cells showing diffuse intracellular NBCe1-A immunoreactivity during acute metabolic acidosis. Values are mean percentages ± SE of 3 independent experiments (\(^* P < 0.05\)). Acute (G) and chronic (H) metabolic alkalosis resulted in a significant decrease of basolaterally NBCe1-A labeled cells, compared with untreated controls (\(^* P < 0.05\) and \(^** P < 0.01\) by 1-way ANOVA and Dunnett’s post hoc test; \(n = 3\)).

Fig. 1. Effect of acid-base disturbances on NH2-terminal electrogenic Na\(^+\)/HCO\(_3\)\(^-\) cotransporter 1 (NBCe1) variant NBCe1-A in rat kidney. Double immunofluorescence on fixed tissue sections with antibodies against NBCe1-A (red) and Na\(^+\)/H\(^+\) exchanger 3 (NHE3; green). A–C: NBCe1-A distribution in rat renal proximal tubule (PT) in controls (A) and during acute metabolic acidosis (B) or alkalosis (C). D–F: NBCe1-A distribution in rat renal PT in controls (D) and during chronic metabolic acidosis (E) or alkalosis (F). G and H: quantification of immunohistochemical data showing increased number of basolaterally stained cells and decreased number of cells with intracellular NBCe1-A immunoreactivity during acute metabolic acidosis. Values are mean percentages ± SE of 3 independent experiments (\(^* P < 0.05\)). Acute (G) and chronic (H) metabolic alkalosis resulted in a significant decrease of basolaterally NBCe1-A labeled cells, compared with untreated controls (\(^* P < 0.05\) and \(^** P < 0.01\) by 1-way ANOVA and Dunnett’s post hoc test; \(n = 3\)).

In chronic controls, 89.7 ± 0.7% of PT cells exhibited basolateral NBCe1-A localization, whereas 9.5 ± 0.6% revealed intracellular NBCe1-A localization (Fig. 1G), confirming previous results (30). During acute metabolic acidosis, 90.6 ± 0.6% of PT cells revealed basolateral NBCe1-A labeling and 9.0 ± 0.4 showed intracellular NBCe1-A distribution (Fig. 1, E and H), thus being similar to the controls. In contrast, chronic metabolic alkalosis had a prominent effect on NBCe1-A localization: 73.8 ± 3.5 and 26.2 ± 3.5% of the rat PT cells showed basolateral and intracellular NBCe1-A immunoreactivity, respectively (Fig. 1, F and H; \(P < 0.01\)).

NBCe1-B distribution in renal PT was not affected by acute acid-base disturbances (Fig. 2, A–C) as shown by representative immunofluorescence labeling of rat renal PT with antibod-
ies against NBCe1-B (red) and NHE3 (green). Similar results obtained by immunohistochemistry with HRP-coupled secondary antibodies were used for quantitative analysis of NBCe1-B distribution. In controls (Fig. 2A), 49.8 ± 1.3% of PT cells exhibited basolateral NBCe1-B immunolabeling, whereas 48.6 ± 1.4% of the cells were intracellularly labeled and in 1.6 ± 0.47% NBCe1-B immunoreactivity was absent (Fig. 2G). An apical NBCe1-B localization in PT could not be detected, demonstrated by the lack of colocalization of NBCe1-B with NHE3 (Fig. 2A). NBCe1-B distribution in renal PT was not affected by acute acid-base disturbances (Fig. 2, B, C, and G). Chronic metabolic acidosis (Fig. 2E) did not affect NBCe1-B in renal PT cells as well. In contrast, during chronic metabolic alkalosis (Fig. 2F), the number of cells with basolateral NBCe1-B immunoreactivity was significantly decreased (38.1 ± 1.3%), compared with the controls (52.8 ± 0.3%, \( P < 0.01 \); Fig. 2H). This was accompanied by a significant increase in the number of cells with intracellular NBCe1-B labeling (Fig. 2H; 60.7 ± 0.9% in acidosis vs. 44.8 ± 0.4% in untreated controls, \( P < 0.01 \)). Absence of NBCe1-B immunoreactivity was observed in 1.1 ± 0.6% of PT cells in chronic alkalotic animals compared with 2.4 ± 0.1% of cells in controls.

The WKPT-0293 Cl.2 cell line expresses key acid-base transporters. To establish an adequate in vitro system that could mimic the in vivo situation, we studied whether the rat renal PT-derived WKPT-0293 Cl.2 cell line (42) meets such prerequisites. First, we addressed the question of whether this cell line retained polarity. Electron microscopy revealed that WKPT-0293 Cl.2 cells exhibited the typical morphological characteristics of renal PT cells, such as a multitude of long microvilli (Fig. 3A, M) at the luminal surface and the deep invaginations of the basal cell surface (Fig. 3B, aster-...
Fig. 3. mRNA expression and cellular localization of acid-base transporters in WKPT-0293 Cl.2 cell line. A–B: ultrastructural analysis of WKPT-0293 Cl.2 cell line. Formation of tight junctions (arrow), presence of microvilli (M) in luminal membrane, and invaginations of basolateral membrane (asterisks) are shown. N, nucleus; LY, lysosome. C: NBCe1-A, vacuolar (V)-ATPase (ε-subunit), NHE3, and NBCe1-B transcripts were detected in WKPT-0293 Cl.2 cells (arrows) by using transporter-specific primers. D: double labeling for NBCe1-A and Na$^{+}$-K$^{+}$-ATPase showed NBCe1-A distribution in cytoplasm and at basolateral plasma membranes of WKPT-0293 Cl.2 cells. E: double immunofluorescence for NHE3 and V-ATPase revealed intracellular V-ATPase immunolabeling and distribution of NHE3 to plasma membrane. F: intracellular and nuclear NBCe1-B distribution in WKPT-0293 Cl.2 cells and plasma membrane localization of housekeeping Na$^{+}$-K$^{+}$-ATPase. G: double immunofluorescence for NHE3 and NBCe1-B showed distinct distribution patterns of these transporters. H and I: NBCe1-A (H) and NBCe1-B (I) protein expression by immunoblotting in homogenates of controls and after extracellular acid-base changes. Immunoblots were probed either with monoclonal antibody against GAPDH or with variant specific NBCe1 antibody. NBCe1-A protein was significantly down-regulated during acute alkalosis (**P < 0.01 after densitometric analysis of signal ratio NBCe1-A:GAPDH and Student’s t-test) but remained unchanged during acute extracellular acidosis. In contrast, NBCe1-B protein level was not changed during acute acidosis or alkalosis. Blots are representative of 3 different experiments. Thirty micrograms of protein was loaded per lane.
isks). In addition, sealing of the intercellular clefts by tight junctions (Fig. 3A, arrow) located on the cell boundaries near the free surface could be observed, demonstrating the expression of polarity at the ultrastructural level.

Characterization of this cell line with regard to key acid-base transporters is shown in Fig. 3, C–G, which illustrates transcript expression and cellular localization of the NH2-terminal NBCe1 variants NBCe1-A and NBCe1-B, as well as of the V-ATPase (ε-subunit) and NHE3. As shown in Fig. 3C, transcripts of the expected size of 344 bp (lane 3), 72 bp (lane 6), 198 bp (lane 8), and 470 bp (lane 10) for NBCe1-A, V-ATPase, NHE3, and NBCe1-B, respectively, could be amplified from WKPT-0293 Cl.2 cell-line cDNA.

Subsequently, subcellular localization of the respective proteins was assessed by immunofluorescence. Intracellular NBCe1-A immunoreactivity (green) of moderate to strong intensity was observed in WKPT-0293 Cl.2 cells (Fig. 3D). In addition, NBCe1-A labeling in the plasma membrane was also detected that partially colocalized with Na+/K+-ATPase (red; Fig. 3D). The membrane-staining pattern was also observed for NHE3 (Fig. 3, E and G; red). In contrast, as illustrated in Fig. 3E by double immunofluorescence, the V-ATPase labeling pattern (green) was found to be punctate and intracellular, whereas nuclei were devoid of both NHE3 and V-ATPase immunoreactivity. In contrast, NBCe1-B immunoreactivity (Fig. 3, F and G; green) was detected intracellularly, both in cytosolic compartments and associated with the nuclear compartment of WKPT-0293 Cl.2 cells. Moreover, double labeling of NBCe1-B with Na+/K+-ATPase (Fig. 3F) or NHE3 (Fig. 3G) clearly showed that these proteins do not colocalize, confirming the in vivo data and demonstrating that NBCe1-B is not expressed in the apical plasma membrane of cultured PT cells.

Effect of acid-base disturbances on protein expression of WKPT-0293 Cl.2 cells. Figure 3H illustrates the Western blot analysis of the WKPT-0293 Cl.2 homogenate from the controls and during acute extracellular acidosis and alkalosis by using anti-GAPDH as a housekeeping gene and NBCe1-A antibodies. NBCe1-A protein expression was comparable between controls and after extracellular acidosis (1.18 ± 0.19-fold; $P = 0.39$) but was downregulated after acute extracellular alkalosis (9.4 ± 3.1-fold; **$P < 0.01$; $n = 3$). In contrast, as shown in Fig. 3I, NBCe1-B protein expression was not significantly affected by extracellular acidosis or alkalosis (0.9 ± 0.1-fold, $P = 0.50$, $n = 3$ and 1.0 ± 0.1-fold, $P = 1.0$, $n = 3$ for acidosis and alkalosis, respectively).

NBCe1-A and NBCe1-B mRNA and protein expression in rat SMG. Figure 4A shows the results obtained with rat SMG cDNA by RT-PCR using NBCe1-A- and NBCe1-B-specific primers. Transcripts of the expected sizes of ~344 bp and ~473 bp for NBCe1-A and NBCe1-B, respectively, were detected (Fig. 4A, lanes 3 and 6 arrows). In the absence of RT enzyme from the reaction, no bands were visible (not shown). The presence of the respective translated proteins in rat SMG was determined in rat SMG homogenate and plasma membrane fraction by immunoblotting using variant-specific antibodies. As shown in Fig. 4B, with the anti-NBCe1-A and anti-NBCe1-B antibody, prominent bands of ~130 kDa and of ~150 kDa, respectively, were labeled in the plasma membrane fraction (Fig. 4B, lanes 2 and 4), whereas in whole SMG homogenate (Fig. 4B, lanes 1 and 3), no bands were visible. With the anti-NBCe1-B antibody, additional bands at ~110 kDa and ~60 kDa were also detected.

Cellular localization of NBCe1-A and NBCe1-B mRNA and protein in rat SMG. Figure 4, C and H, illustrate cellular localization of NBCe1-A and NBCe1-B mRNA, respectively, in paraffin sections of PLP-fixed rat SMG determined by in situ hybridization. With the NBCe1-A-specific probe (Fig. 4C), striated duct cells exhibited intracellular staining of strong intensity, representing NBCe1-A mRNA expression. In contrast, granular ducts were weakly labeled, and acinar cells were devoid of NBCe1-A mRNA. Staining was considered specific, because labeling of duct cells was absent when sections were incubated with the respective sense probe (Fig. 4D). Figure 4H shows the labeling pattern of paraffin sections of PLP-fixed rat SMG treated with the NBCe1-B antisense probe. A similar labeling pattern to that obtained for NBCe1-A mRNA was observed. Cells lining the striated and granular ducts exhibited diffuse intracellular staining. Sections incubated with the respective sense probe revealed no detectable labeling, as illustrated in Fig. 4I.

Subcellular localization of NBCe1-A and NBCe1-B protein was assessed by immunohistochemistry. Figure 4E exemplifies the labeling pattern on cryosections of rat SMG treated with the anti-NBCe1-A antibody (dilution 1:100) by using immunofluorescence light microscopy. Diffuse intracellular NBCe1-A immunoreactivity of moderate intensity was observed in striated duct cells but was absent in acinar cells. Intriguing was the observation that in interlobular ducts (Fig. 4F) and main excretory duct cells, NBCe1-A immunolabeling was present in the luminal plasma membrane. Incubation of the fixed sections with anti-NBCe1-A antibody in the presence of the peptide antigen (1 mM) abolished NBCe1-A labeling, as shown in Fig. 4G. Figure 4J illustrates staining obtained in the rat SMG by using the specific anti-NBCe1-B antibody. Prominent immunoreactivity could be detected at the basolateral-cell side of striated duct cells, representing labeling of the basolateral cell membrane. Again, in acinar cells NBCe1-B immunoreactivity was absent. No differences of NBCe1-B immunoreactivity could be detected between different duct segments. NBCe1-B staining was considered specific because immunolabeling was abolished in sections that had been incubated with preimmun serum, as shown in Fig. 4K.

Effect of acute and chronic acid-base disturbances on NBCe1-A and NBCe1-B localization in duct cells of rat SMG. NBCe1-A and NBCe1-B distribution after acute and chronic metabolic acid-base changes was assessed by counting striated duct cells exhibiting basolateral, apical, or intracellular NBCe1-A and NBCe1-B immunoreactivity in controls and in rats in which acute or chronic metabolic acidosis and alkalosis was induced.

NBCe1-A distribution was not altered by chronic metabolic alkalosis (Fig. 5, C and J) or by acute acid-base disturbances (data not shown). In contrast, NBCe1-A distribution in striated and intralobular duct cells of rat SMG was affected by chronic metabolic acidosis. As shown in Fig. 5, A and J, in controls, 85.6 ± 2.1 and 14.4 ± 2.1% of striated duct cells exhibited intracellular or basolateral NBCe1-A localization, respectively. Chronic metabolic acidosis significantly increased the number of basolaterally stained striated duct cells to 34.7 ± 1.8% ($P < 0.01$). Consequently, the number of striated duct cells revealing intracellular NBCe1-A immunoreactivity was significantly de-
creased (Fig. 5, B and J; 65.3 ± 1.8%; P < 0.01) compared with the controls, indicating NBCe1-A recruitment from the cytoplasm toward the basolateral membrane of striated duct cells. During chronic metabolic acidosis, NBCe1-A underwent an adaptive redistribution in main duct cells of rat SMG as well. As illustrated in Fig. 5, D and E, in controls, 62.8 ± 3.8% of the main duct cells showed apical NBCe1-A localization and 37.2 ± 3.8% of the cells revealed an intracellular NBCe1-A distribution. Chronic acid load resulted in a significant decrease of apically labeled main duct cells (44.8 ± 5.1%; P < 0.05), accompanied by a significant increase in the number of cells exhibiting intracellular NBCe1-A distribution (Fig. 5, E and K; 55.2 ± 5.1%; P < 0.05), suggesting NBCe1-A retrieval from the apical membrane to the cytoplasm. In contrast, NBCe1-A distribution in main duct cells was comparable between chronic alkalotic animals and controls (Fig. 5, F and K).

Acute metabolic acidosis affected NBCe1-B distribution in duct cells of rat SMG as shown in Fig. 5, G and H. In controls, 71.4 ± 1.7% of striated duct cells exhibited basolateral NBCe1-B localization, whereas 28.6 ± 1.7% of the cells revealed an intracellular distribution pattern (Fig. 5G). During acute metabolic acidosis, however (Fig. 5H), the number of basolaterally stained striated duct cells was dramatically decreased to 3.8 ± 0.1% (P < 0.0001) compared with the controls. Consequently, the number of cells showing intracellular NBCe1-B immunoreactivity was significantly increased compared with the controls (96.2 ± 0.1%; P < 0.0001; Fig. 5L), suggesting a redistribution of NBCe1-B from the basolateral membrane toward the cyto-

Fig. 4. NBCe1-A and NBCe1-B in rat submandibullar gland (SMG). A: RT-PCR analysis with specific NBCe1-A and NBCe1-B primers revealed specific transcript expression in rat SMG (arrows). B: immunoblotting of rat SMG homogenate (Ho) and plasma membrane (PM) fractions with specific antibodies against NH2-terminal NBCe1 variants. Arrows point to bands of expected size; 50 μg protein was loaded per lane. One representative blot of five experiments is shown. Mr, apparent molecular mass. C and H: cellular distribution of NBCe1-A and NBCe1-B mRNA in rat SMG. NBCe1-A (C) and NBCe1-B (H) mRNA were exclusively localized in duct cells (GD, granular ducts; SD, striated ducts), as shown by in situ hybridization. Incubation of sections with sense probe (D, I) revealed no labeling. E: striated duct cells (SD) of rat SMG showed diffuse intracellular NBCe1-A immunoreactivity. F: interlobular (ILD) and main duct cells (not shown) demonstrated a prominent luminal NBCe1-A distribution. G: labeling was abolished in sections incubated with anti-NBCe1-A antibody that had been preabsorbed with 1 mM peptide antigen. J: basolateral NBCe1-B immunolabeling was found in striated duct cells (SD) but was absent in acinar cells (A) of rat SMG. K: no labeling could be observed in sections incubated with preimmune serum.
Fig. 5. Cellular distribution of NBCe1-A and NBCe1-B in SMG of control rats and following acute or chronic metabolic acidosis and alkalosis. Chronic metabolic acidosis induced a significant increase in number of striated duct cells (SD) with basolateral NBCe1-A immunostaining (B) compared with controls (A) and was associated with a significant decrease in number of intracellularly labeled duct cells. A, acinar cells. Chronic metabolic alkalosis (C) had no effect on NBCe1-A distribution. D: NBCe1-A distribution in main duct cells (MD) of controls. E: adaptive redistribution of NBCe1-A in main duct cells of rat SMG during chronic metabolic acidosis. A decrease in number of luminally stained cells and an increase in number of cells showing intracellular NBCe1-A localization was observed. F: during chronic metabolic alkalosis, no changes on NBCe1-A distribution in main duct cells were observed compared with controls. G–I: NBCe1-B distribution in salivary ducts during acute metabolic acid-base changes. During acute metabolic acidosis, a redistribution of NBCe1-B in striated duct cells (SD) occurred from basolateral localization in controls (G) toward an intracellular labeling pattern (H). In contrast, acute metabolic alkalosis had no effect on NBCe1-B immunolocalization (I). J–L: quantification of data revealed statistically significant differences from controls. Data are presented as mean percentage ± SE from 3 independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.0001 by using 1-way ANOVA and Dunnett’s post hoc test.
plasm. In contrast, NBCe1-B localization was neither affected by acute metabolic alkalosis (Fig. 5J) nor by chronic metabolic acid-base disturbances (data not shown).

DISCUSSION

In the present study, cellular distribution of the NH2-terminal NBCe1 variants NBCe1-A and NBCe1-B were examined under physiological conditions and after acute or chronic systemic acid-base perturbations in rat kidney and SMG in vivo. Previous functional and morphological studies have shown NBCe1 expression to be confined to the PT in the rat kidney (27, 35) and to duct cells in the rat SMG (22, 31). We have tested the hypothesis that redistribution of NBCe1 is a posttranslational regulatory mechanism during acid-base disturbances. In addition, while we focused on the role of NBCe1 variants NBCe1-A and NBCe1-B, the impact of metabolic acidosis and alkalosis on a HCO3\(^{-}\)-secreting epithelium (salivary ducts) has been investigated compared with a HCO3\(^{-}\)-reabsorbing epithelium (renal PT).

Besides its function as an HCO3\(^{-}\)-reabsorbing epithelium, the renal PT is involved in maintenance of systemic acid-base balance. The electrogenic NBCe1-A is localized to the basolateral membrane, operates as an acid loader, and is responsible for HCO3\(^{-}\) reabsorption in this nephron segment (7, 26). Along this line, NBCe1-deficient mice or patients with mutations of the SLC4A4 gene suffer from severe renal metabolic acidosis (14, 16–18). However, besides NBCe1-A, the cells of the renal PT express NBCe1-B as well, whose physiological significance, however, is not clear (13, 30). In the present study, NBCe1-A showed basolateral distribution, whereas NBCe1-B revealed intracellular localization, together with labeling of the basolateral membrane (Figs. 1A and 2A). These data confirm previous observations from our laboratory (30) and by Schmitt et al. (35), who have used an antibody against a common epitope for both NBCe1 variants. In contrast, Endo et al. (13) showed luminal localization of NBCe1-B in PT. The reason for this discrepancy may be attributed to the use of different antibodies or processing for immunohistochemistry.

Previous studies have shown intriguing similarities between salivary glands and kidney regarding the distribution of acid-base transporters in response to acid or alkalai load. Both duct cells of rat SMG and renal intercalated cells express the V-ATPase, whose localization undergoes an adaptive redistribution during metabolic acid-base disturbances (6, 32, 33). NHE3 is expressed in duct cells of rat SMG and renal PT as well and shows downregulation during alkalai load in both epithelia (24, 44). Although NBCe1 has been functionally and morphologically described in duct cells of human and rat salivary glands (22, 29, 31), the expression and cellular distribution of the NH2-terminal NBCe1 variants had not been investigated so far. The results of the present study provide the first evidence for mRNA and protein expression of NBCe1-A and NBCe1-B in rat SMG (Fig. 4). These data extend previous observations by using an antibody raised against a COOH-terminal sequence of NBCe1 (31) and are in agreement with the notion that NBCe1-A and NBCe1-B are not exclusively expressed in HCO3\(^{-}\)-reabsorbing or -secreting epithelia, respectively, but are coexpressed in these tissues. Indeed, NBCe1-A and NBCe1-B coexpression has also been reported in pancreas, intestine, and eye (8, 34, 36). NBCe1-B was predominantly localized in the basolateral membranes of striated (Fig. 4J) duct cells, consistent with previous immunohistochemical and functional studies in the salivary glands and other HCO3\(^{-}\)-secreting epithelia, such as pancreas (30) or intestine (36), and suggesting a contribution of NBCe1-B to HCO3\(^{-}\) uptake in the basolateral membrane, in addition to or alternatively to the transport pathways involving functional coupling of Na\(^{+}\)/H\(^{+}\) and Cl\(^{-}\)/HCO3\(^{-}\) exchangers. NBCe1-A immunolabeling was diffusely distributed (Fig. 4E) in striated duct cells, reflecting a cytoplasmic vesicular pool containing NBCe1-A, which can be recruited to the plasma membrane depending on the physiological stimulus. In interlobular (Fig. 4F) and main (Fig. 5D) duct cells, NBCe1-A exhibited a prominent apical NBCe1-A localization, matching previous observations (30, 31, 40). The physiological relevance of the apical NBCe1-A localization is challenging, because the stoichiometry of the transporter in salivary duct cells has not been determined so far. Ductal luminal NBCe1-A with a stoichiometry of 1 Na\(^{+}\):3 HCO3\(^{-}\) acting together with basolateral NBCe1-B operating with a stoichiometry of 1 Na\(^{+}\):2 HCO3\(^{-}\) could lead to HCO3\(^{-}\) efflux and transepithelial HCO3\(^{-}\) transport. Notably, luminal NBCe1-B has been recently demonstrated in functional studies in guinea pig salivary glands (21).

Early functional studies have shown adaptive changes of Na\(^{+}\)/HCO3\(^{-}\) cotransport activity in renal PT during acid-base disturbances (3, 25, 37). However, more recent studies failed to record differences in the mRNA (7) or protein levels (5, 13, 19) of NBC1 in acidic animals that could account for the observed increased NBC activity. In addition, acid-base changes affect not only the kidney but other epithelia as well. Here we show that redistribution of NBCe1-A and NBCe1-B is a potential posttranslational regulatory mechanism during acid-base disturbances. However, the impact of metabolic acid-base changes was different in the HCO3\(^{-}\)-reabsorbing PT compared with the HCO3\(^{-}\)-secreting salivary ducts.

Acute acid-base disturbances affected NBCe1-A but not NBCe1-B distribution in rat PT. NBCe1-A underwent redistribution toward the basolateral membrane during acute metabolic acidosis and toward the cytosol during acute metabolic alkalosis, consistent with a role of renal PT in the regulation of systemic acid-base homeostasis (Fig. 1) and in agreement with early functional studies that have demonstrated increased Na\(^{+}\)-HCO3\(^{-}\) cotransporter activity during metabolic acidosis and decreased activity during metabolic alkalosis (3, 25, 37). Interestingly, chronic metabolic acidosis had no effect on NBCe1-A or NBCe1-B distribution, suggesting that regulation of chronic acidosis was taken over by other transporters, such as the NHE3 in the PT or the V-ATPase in the collecting duct, as previously suggested (4, 6, 9, 44). These data are consistent with previous observations that have shown no differences in total NBCe1 mRNA (7) or protein (5, 19) in the kidney of chronic metabolic acidotic rats compared with controls. In contrast, chronic metabolic alkalosis caused a redistribution pattern of NBCe1-A and NBCe1-B favoring decreased HCO3\(^{-}\) reabsorption in the PT (Figs. 1H and 2H), in accordance with functional studies (3).

Opposite to the results obtained in renal PT, acute acid-base disturbances affected NBCe1-B but not NBCe1-A in striated ducts of SMG. Acute metabolic acidosis induced a redistribution of NBCe1-B in duct cells of SMG from the basolateral membrane toward the cytosol. A similar redistribution pattern
was also observed in another HCO₃⁻-secretory epithelium, the main pancreatic duct (data not shown), implicating that our observations likely reflect regulation of NBCe1-B in HCO₃⁻-secretory epithelia during acidosis, rather than a salivary duct-specific response. Chronic metabolic acidosis recruited NBCe1-A from the cytosol of striated duct cells toward the basolateral membrane and from the apical membrane toward the cytosol in main duct cells (Fig. 5E).

These data show that the response of the renal PT and the salivary duct epithelium to systemic acid-base disturbances consists of an opposite recruitment pattern for NBCe1-A and NBCe1-B, reflecting their specialized functions as HCO₃⁻-reabsorbing and HCO₃⁻-secretory epithelium, respectively. Moreover, the results propose redistribution of the transporters as a potential posttranslational regulation modus during acid-base disturbances and highlight the complexity and fine tuning of the regulation of these transporters during acid-base disturbances in vivo.

Finally, the in vivo data in the kidney were additionally confirmed and extended in vitro by using the immortalized rat kidney PT cell line WKPT-0293 Cl.2 (42). This cell line has been a useful model to study Cd₂⁺ transport and mechanisms of apoptosis (1, 20, 39, 41). Here we showed that this cell line has retained polarity and expresses relevant H⁺ and HCO₃⁻ transporters at both the mRNA and protein levels (Fig. 3). Partial colocalization with Na⁺-K⁺-ATPase demonstrated expression of NBCe1-A at the plasma membrane, whereas NBCe1-B was found intracellularly distributed and showed no colocalization with NHE3, supporting the in vivo data and against the notion of apical NBCe1-B expression. Induction of extracellular acidosis or alkalosis had no effect on NBCe1-B protein expression (Fig. 3). Extracellular alkalosis, however, downregulated NBCe1-A protein (Fig. 3H) in WKPT-0293 Cl.2 cells. This observation could be specific to cell lines and indicates that under in vitro conditions, alkalotic stress and/or the turnover of membrane proteins is higher than in vivo or that NBCe1-A is regulated in different ways than in vivo under alkalotic conditions. Nevertheless, this cell line can mimic the in vivo situation and can therefore be considered as an adequate tool to study regulation of acid-base transporters in the kidney.

REFERENCES


