Basolateral Na^+-dependent HCO_3^- transporter NBCn1-mediated HCO_3^- influx in rat medullary thick ascending limb

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The electroneutral Na^+-dependent HCO_3^- transporter NBCn1 is strongly expressed in the basolateral membrane of rat medullary thick ascending limb cells (mTAL) and is up-regulated during NH_4^+-induced metabolic acidosis. Here we used *in vitro* perfusion and BCECF video-imaging of mTAL tubules to investigate functional localization and regulation of Na^+-dependent HCO_3^- influx during NH_4^+-induced metabolic acidosis. Tubule acidification was induced by removing luminal Na^+ (ΔpH_i: 0.88 ± 0.11 pH units, $n = 10$). Subsequently the basolateral perfusion solution was changed to CO_2/HCO_3^- buffer with and without Na^+. Basolateral Na^+–H^+ exchange function was inhibited with amiloride. Na^+-dependent HCO_3^- influx was determined by calculating initial base flux of Na^+-mediated re-alkalinization. In untreated animals base flux was 8.4 ± 0.9 pmol min^{-1} mm^{-1}. A 2.4-fold increase of base flux to 21.8 ± 3.2 pmol min^{-1} mm^{-1} was measured in NH_4^+-treated animals (11 days, $n = 11$). Na^+-dependent re-alkalinization was significantly larger when compared to control animals (0.38 ± 0.03 *versus* 0.22 ± 0.02 pH units, $n = 10$). In addition, Na^+-dependent HCO_3^- influx was of similar magnitude in chloride-free medium and also up-regulated after NH_4^+ loading. Na^+-dependent HCO_3^- influx was not inhibited by 400 μm DIDS. A strong up-regulation of NBCn1 staining was confirmed in immunolabelling experiments. RT-PCR analysis revealed no evidence for the Na^+-dependent HCO_3^- transporter NBC4 or the two Na^+-dependent Cl^-/HCO_3^- exchangers NCBE and NDCBE. These data strongly indicate that rat mTAL tubules functionally express basolateral DIDS-insensitive NBCn1. Function and protein are strongly up-regulated during NH_4^+-induced metabolic acidosis. We suggest that NBCn1-mediated basolateral HCO_3^- influx is important for basolateral NH_3 exit and thus NH_4^+ excretion by means of setting pH_i to a more alkaline value.

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The thick ascending limb of Henle's loop (TAL) serves a number of important transport functions. On the one hand it is critically involved in NaCl absorption, generation of high interstitial osmolality and thus the countercurrent system of urine concentration (Greger, 1985). In addition it is involved in the regulation of acid–base homeostasis. Up to 15% of HCO_3^- is absorbed in TAL, all of which appears dependent on the presence of the luminal Na^+–H^+ exchanger NHE3 (Good *et al.* 1984; Good, 1985). Subsequently mTAL is involved in the excretion of NH_4^+. NH_4^+ entering the loop of Henle is able to travel transcellularly and uses a 'medullary short cut' to eventually appear in the collecting duct (Good, 1994).

Recently an electroneutral Na^+-dependent HCO_3^- cotransporter (NBCn1) was cloned (Choi *et al.* 2000) and immunolabelling demonstrated its presence in basolateral membrane domains of thick ascending limb in rat kidney outer medulla (Vorum *et al.* 2000). The labelling was more pronounced in the inner stripe of the outer medulla (ISOM), somewhat less in the outer stripe of the outer medulla and absent in the cortex (Vorum *et al.* 2000). An electroneutral Na^+-dependent HCO_3^- transporter with a proposed stoichiometry of 1 Na^+ and 1 HCO_3^- will function as a HCO_3^- importer (Choi *et al.* 2000).

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Indeed, basolateral Na^+-dependent HCO_3^- influx was identified recently in isolated perfused rat medullary thick ascending limbs (Bourgeois *et al.* 2002). Apparently, this is in conflict with the need for basolateral HCO_3^- extrusion during HCO_3^- absorption (Good *et al.* 1984; Good, 1985) As such, the functional significance of a basolateral Na^+-dependent HCO_3^- importer remains undefined. A subsequent study from our group identified that NBCn1 protein is strongly up-regulated in a rat model of chronic metabolic acidosis induced by either NH_4^+ feeding or inclusion of NH_4^+ into the drinking water (Kwon *et al.* 2002). Thus we have speculated that NBCn1 may play a significant role in the excretion of NH_4^+ (Kwon *et al.* 2002). NH_4^+ that has entered the mTAL cell via the furosemide (frusemide)-sensitive NKCC2 transporter or the ROMK channel will dissociate into NH_4^+ and NH_3. The generated NH_3 will leave the cell via non-ionic diffusion preferentially over the basolateral membrane and finally is transported into the acidic compartment of the collecting duct. The remaining proton may either be transported directly via a Na^+–H^+ antiporter and/or could be buffered by import of HCO_3^-. The generation of CO_2 would subsequently allow for the recycling of HCO_3^- over the basolateral membrane. In this hypothesis the extrusion of a proton/import of a HCO_3^- ion serves to set pH_i to more alkaline values, a state where, for example, NH_3 extrusion but also NaCl absorption is less restrained. Thus the purpose of this study was to functionally localize Na^+-dependent HCO_3^- uptake in the intact, isolated perfused mTAL tubules. Furthermore it was intended to investigate if basolateral Na^+-dependent HCO_3^- uptake is functionally up-regulated in rats treated with added NH_4^+ in their drinking water, a well-known condition for up-regulated NBCn1 expression (Kwon *et al.* 2002).

Methods

Tubule perfusion

All handling and use of the animals complied with Danish animal welfare regulations. Experiments were carried out using 4- to 6-week-old female Wistar rats weighing 70–80 g. The animals were divided into two age-matched groups. The control group had free access to food (standard rat laboratory diet, Altromin, Lage, Germany) and tap water. Also in the experimental group the animals had free access to food and water. Their drinking water contained 0.196 M ammonium chloride as previously described (Kwon *et al.* 2002). Animals were killed after 8.0 ± 1.4 days ($n = 11$) by decapitation, and the left or right kidney was removed rapidly, placed in ice-cold Ringer solution (see below) and subsequently sliced as previously described (Wright *et al.* 1990). Kidney slices were transferred into a dissection chamber continuously cooled at 4°C and gassed with carbogen (5% CO_2–95% O_2). Medullary thick ascending limbs (mTAL) were dissected from the inner stripe of the outer medulla using ultrafine watchmaker forceps. The kidney tubules were transferred into a specialized perfusion chamber mounted on an inverted microscope. Isolated tubules were perfused using a system of concentric glass pipettes previously used and developed by R. F. Greger and W. Hampel (Greger & Hampel, 1981).

Digital video imaging

The set-up consisted of an inverted microscope (Axiovert 100 TV, Zeiss, Jena, Germany) with a ×63 objective (C-Apochromat ×63, 1.2 water, Zeiss, Jena, Germany), a monochromator (Polychrome IV, Till Photonics, Planegg, Germany) and a digital camera (MicroMax, 5 MHz, Prinction Instruments, NJ, USA). Image acquisition and data analysis were performed with the software package Metamorph/Metafluor (Universal Imaging, West Chester, PA, USA). Freshly dissected mTAL were mounted into the perfusion system (Greger & Hampel, 1981). Measurement of pH_i was performed with the 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF). Tubules were incubated in 20 μM basolateral BCECF/AM for 20 min at room temperature in control solution (No. 1, see Table 1). As a measure of pH_i the fluorescence emission ratio at 488 nm/436 nm excitation was used and the recording speed was 1–2 Hz. Care was taken to reduce cellular damage induced by excitation light (Weiner & Hamm, 1989). This was accomplished by using neutral grey filters in the excitation light path and a 4-fold binning function of the imaging system. In each experiment the fluorescence signal was recorded from the entire tubule. The average length of the perfused tubule was around 300 μm. During the dye-loading period the tubule was continuously perfused from the luminal side with solution 1. The experiment was started 5–10 min after washout of extracellular dye and after a stable fluorescence ratio was reached. Depending on the viability of the individual tubule, stable BCECF 488 nm/436 nm ratios could be measured for 45 min or more.

BCECF signals were calibrated using the high K^+–nigericin method at the end of the experiment (Thomas *et al.* 1979). The following calibration solution previously used by others for the same tubule segment (Watts *et al.* 1994) was used and contained (mM): KCl 95, NaH_2PO_4 0.4, Na_2HPO_4 1.6, glucose 5, $MgCl_2$ 1, calcium gluconate 1.3, Hepes 25, *N*-methyl-D-glucamine (NMDG) 20 and NaCl 15.

Table 1. Experimental solutions

Component	No. 1: Hepes HCO_3^- free	No. 2: Hepes Na^+ free HCO_3^- free	No. 3: HCO_3^-	No. 4: HCO_3^- Na^+ free	No. 5: HCO_3^- Cl^- free	No. 6: HCO_3^- Cl^- free, Na^+ free	No. 7: Hepes Cl^- free, Na^+ free
NaCl	145	—	118	—	—	—	—
NMDG	—	145	—	120	—	119	145
KH_2PO_4	0.4	0.4	0.4	0.4	0.4	0.23	0.4
K_2HPO_4	1.6	1.6	1.6	1.6	1.6	0.95	1.6
Glucose	5	5	5	5	5	5	5
Hepes	5	5	5	5	5	5	5
$MgCl_2$	1	1	1	1	—	—	—
$MgSO_4$	—	—	—	—	—	6	—
Ca gluconate	1.3	1.3	1.3	1.3	8	4	4
EDTA	—	—	—	—	—	0.026	—
$NaHCO_3^-$	—	—	24	—	24	—	—
HCl	—	130	—	100	—	—	—
Choline-HCO_3^-	—	—	—	24	—	24	—
Na gluconate	—	—	—	—	121	—	—
Gluconic acid	—	—	—	—	—	121	145

All solutions had a pH of 7.40 prior to experiments. All solutions were gassed with carbogen (5%CO_2–95%O_2) except for solutions 1 and 7.

Measurement of intracellular buffering capacity

Quantification of HCO_3^- transport requires the knowledge of intracellular buffering capacity β_i. We therefore measured the intracellular buffering capacity in normal and NH_4^+-treated animals using a similar protocol to that described by D. Good (Watts *et al.* 1994). To minimize the effect of HCO_3/CO_2 buffering and to block Na^+-dependent pH_i regulatory mechanisms Na^+-free Hepes-containing solution (No. 1) was used on both sides of the tubule. In addition 5 mM Ba^{2+}, 100 μM furosemide and 1 mM ouabain were added to the bath. Subsequent addition of 2.5 mM trimethylamine to the bath rapidly increased mTAL pH_i by 0.26 ± 0.03 units in normal and 0.19 ± 0.02 units in NH_4^+-treated rats. β_i was calculated as $\Delta[HB^+]/\Delta pH_i$, where ΔpH_i is the increase in pH_i resulting from weak base addition and $\Delta[HB^+]$ is the change in intracellular trimethylammonium concentration, calculated from its pK_a (9.8 at 37°C) and assuming that the concentration of trimethylamine base is equal in intracellular and extracellular fluids at steady state. pH_i values in the experiments for measuring β_i corresponded closely to pH_i values in the experiments in which Na^+-dependent HCO_3^- influx was quantified (pH_i 6.4). In normal rat tubules β_i was calculated to be 98 ± 16 mM ($n = 5$), which was not significantly different from 114 ± 12 mM ($n = 8$) found in tubules from NH_4^+-treated animals.

Calculation of base flux

Base flux (J_{base}) (in pmol min^{-1} mm^{-1}) was calculated using the following equation: $J_{base} = \Delta pH_i/\Delta t \times \beta_{total} \times V$, where $\Delta pH_i/\Delta t$ is the initial rate of pH_i change (in pH units min^{-1}), β_{total} is the total cellular buffering capacity (in mM (pH unit)$^{-1}$ l^{-1}), and V is the cell volume (in nl) per 1 mm of tubule length. pH_i changes were determined by a linear fit of the pH_i rise during the first 10–20 s after re-addition of Na^+. To account for the increased buffering capacity in CO_2/HCO_3^--containing solutions β_{total} was calculated as $\beta_i + 2.3 \times [HCO_3^-]_i$, where $[HCO_3^-]_i$ is the intracellular HCO_3^- concentration (Roos & Boron, 1981). $[HCO_3^-]_i$ was calculated as follows: $[HCO_3]_i = 0.03\ P_{CO2} \times 10^{(pHi-6.1)}$. In carbogen-gassed solutions, P_{CO2} was assumed to be 37 mmHg. V (0.32 nl mm^{-1}) was calculated by measuring the dimensions of the tubule (volume = (radius of the tubule)^2 $\times$ π $\times$ length) with subtraction of the volume of the tubule lumen. A positive (J_{base}) value indicates a net base influx.

Solutions and chemicals

All experimental solutions not mentioned directly in the text are listed in Table 1. BCECF was obtained from Molecular Probes (Eugene, OR, USA). All other chemicals were of the highest grade of purity available and were obtained from Sigma-Aldrich Denmark (Vallensbæk Strand) and Merck (Darmstadt, Germany).

Immunohistochemistry

Kidneys from six NH_4Cl-loaded rats and six control rats were fixed by retrograde perfusion via the aorta with 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4)

Table 2. Primers used to identify NBCn1, NBC4, NCBE, NDCBE, NKCC2 and BTR1 transcripts

Transporter		Primer (5′–3′)	Gene accession number
NBCn1*	F	GACTCCATAAGGGAGAATGTTCGA	AF080106 (rat)
A-cassette	R	TCACCACTTTTACTACTGTCCAGG	
NBCn1**	F	CAAGCTCATGGATCTGTGC	AF080106 (rat)
B-cassette	R	ACTCACAGGCTTTTCAGGG	
NBC4	F	ATGGAGAGCTTCCTGGGCAC	AF243499 (human)
	R	CTCAGCAGAGACCAGTCCAG	
NCBE	F	GCAGGTCAGGTTGTTTCTCCTC	NM_022058 (human)
	R	TCTTCCTCTTCTCCTGGGAAGG	
NDCBE	F	GCTCAAGAAAGGCTGTGGCTAC	NM_004858 (human)
	R	ACGCCTTAATGACCCAGAGCAG	
BTR1	F	CACCTGCTGTCAGATACCATCC	AF336127 (human)
	R	TGGTGAGCAGCTGTCTCTGATG	
NKCC2	F	CAGTGGTGCCAGTCGTTTCC	NM_019134 (rat)
	R	TGGTGTTGTGGCCAAAGGTT	

*These primers were designed to be able to recognize if NBCn1 transcripts contain exon 7 (with 369 bp) and the so-called 'A-cassette' (42 bp) (Choi *et al.* 2000). **These primers were designed to be able to recognize if NBCn1 transcripts contain the so-called 'B-cassette' (108 bp) (Choi *et al.* 2000). F, forward; R, reverse primers.

and postfixed for 30 min in the same fixative. Kidney slices containing all kidney zones were dehydrated and embedded in paraffin. The paraffin embedded tissues were cut at 2 μm on a rotary microtome (Leica, Germany). The sections were dewaxed in xylene followed by rehydration to 99% and 96% ethanol. At this point, the sections were incubated in 0.3% H_2O_2 in methanol to block endogenous peroxidase activity. After a rinse in 96% ethanol, the sections were rehydrated using 70% ethanol and finally water. To reveal antigens, sections were placed in 1 mM Tris buffer (pH 9.0) supplemented with 0.5 mM EGTA (3,6-di-oxa-octa-methylene-di-nitrilo-tetra-acetic acid) and heated in a microwave oven for 10 min. Non-specific binding of immunoglobulin was prevented by incubating the sections in 50 mM NH_4Cl for 30 min followed by blocking in PBS supplemented with 1% BSA, 0.05% saponin and 0.2% gelatine. Sections were incubated overnight at 4°C with NBCn1 antibodies diluted in 10 mM PBS (pH 7.4) containing 0.1% Triton X-100 and 0.1% BSA. Subsequently, the sections were incubated with horseradish peroxidase-linked goat anti-rabbit secondary antibodies (P448, DAKO Glostrup, Denmark). The labelling was visualized by the diaminobenzidine (DAB) technique and the sections counterstained using Mayer's haematoxylin.

The previously described antibodies against NBCn1 (Vorum *et al.* 2000) and NBCe1 (anti-rkNBC1-CT15) (Maunsbach *et al.* 2000) were used in this study.

RT-PCR from microdissected mTAL segments

Using a commercially available kit (Trizol reagent, Invitrogen Life Technology, USA) total RNA was isolated from 10 microdissected mTAL segments (approx. 2 mm of renal tubule). Isolated total RNA was transcribed into cDNA using the RETROscript. Kit (Ambion, TX, USA). The primers shown in Table 2, derived from cloned rat or human sequences, were used to identify NBCn1, NBC4, NCBE, NDCBE, NKCC2 and BTR1 transcripts. Primers derived from human cDNA were aligned with the corresponding rat genomic sequences (http://www.ensembl.org). Thus the human and rat sequences were identical for the chosen regions of interest.

Statistics

The data shown are either original traces or mean values ± S.E.M. (n), where n refers to the number of experiments. Paired or unpaired t tests were used to compare mean values within or between experimental series. A P value of <0.05 was taken to indicate statistical significance.

Results

Immunolabelling of NBCn1 in normal and NH_4^+-treated young rats

The preceding study identified a strong up-regulation of NBCn1 protein in 'older' rats (mean body weight 250 g) treated with NH_4^+ (Kwon *et al.* 2002). Since successful microdissection of intact single isolated mTAL tubules is critically dependent on the age of the animal, it was necessary to choose significantly younger rats for this study. Tubule dissection in young rats weighing between 70 and 80 g renders sufficient access to single nephron segments, whereas this it is profoundly more

difficult in older animals. We thus repeated the initial immunolabelling experiments in young rats (weighing 70–80 g). Labelling of NBCn1 was compared in untreated and treated young rats (70–80 g). Treated rats received NH_4^+ in their drinking water for 8 days (see Methods). The previously measured NH_4^+-induced metabolic acidosis (Kwon *et al.* 2002) was confirmed by measuring urine pH which was 7.04 ± 0.18 units in normal and 5.9 ± 0.08 units in NH_4^+-treated animals ($n = 6$). As apparent from Fig. 1 there was a very strong up-regulation of NBCn1 labelling in the inner stripe of the outer medulla (ISOM) confirming the observation in adult rats (Kwon *et al.* 2002).

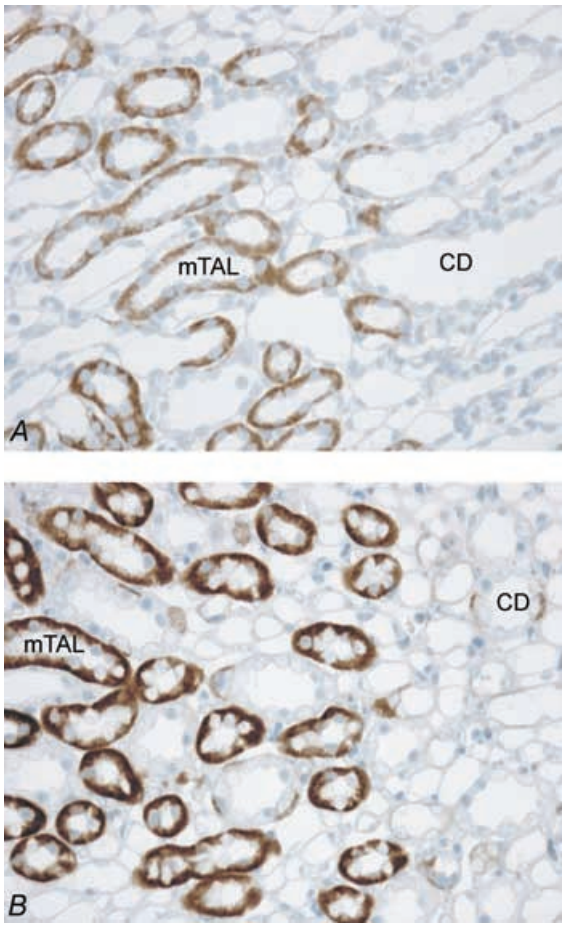

Figure 1. Immunohistochemical localization of the electroneutral Na^+-dependent HCO_3^- transporter NBCn1 in control and NH_4Cl-loaded rats
NBCn1 was localized to the basolateral plasma membrane domains of mTAL segments in the inner stripe of the outer medulla and to the basolateral membrane of intercalated type A cells in collecting ducts. In comparison to controls *(A),* NH_4Cl-loaded rats *(B)* showed a marked increase of NBCn1 labelling. mTAL, medullary thick ascending limb; CD, collecting duct.

RT-PCR analysis

NBCn1 as isolated from a smooth muscle library was shown to represent three different variants (NBCn1B, C or D) (Choi *et al.* 2000). The longest variant, NBCn1D, is composed of 1254 amino acid (AA) residues (3762 bp) (Fig. 2*D*). The two shorter variants lack either a so-called 'A-cassette' (14 AAs, NBCn1C) or the 'B-cassette' (36 AAs, NBCn1B). Close similarity was found to a human clone isolated from skeletal muscle. The authors assigned the name NBC3 to this isoform (Pushkin *et al.* 1999) (accession

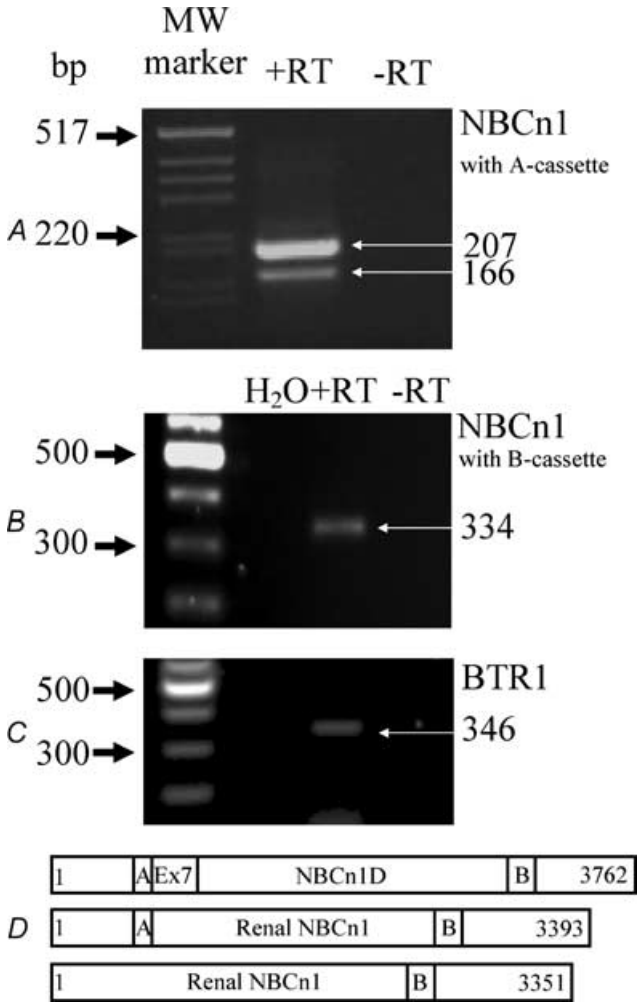

Figure 2. RT-PCR characterization of NBCn1 message extracted from 10 single mTAL tubule segments
Two different primer sets were used (Table 2) to investigate which splice variant is expressed in rat mTAL. The longer transcript in *A* (207 bp) reflects a message which does not contain the information of exon 7 but that of the so-called 'A-cassette'. The shorter amplicon in *A* does not contain the A-cassette. The second primer set (*B*, Table 2) identified the presence of the so-called 'B-cassette' in renal NBCn1. In *D* the full length NBCn1 and the two different renal splice variants are depicted schematically. Also seen in Fig. 3*C* are data showing the specific message for BTR1 in single isolated mTAL tubules.

no. NM-003615). In a subsequent publication, the same group also identified a human kidney isoform of skeletal NBC3 termed NBC2b (accession no. AF089726). This isoform is lacking a segment of 369 bp corresponding to the information encoded in exon 7. Similar results (Fig. 2*A*) are presented here for the rat transcript of NBCn1. One set of primers was designed to recognize if the expected transcript contains exon 7 (bp 964–1332 in NBC1nD, gene accession number AF080106) and the 'A-cassette'. Our amplified cDNA from isolated mTAL tubules was near to 207 bp in size, as expected for a message without exon 7 but containing the 'A-cassette'. RT-PCR results with the same primers from heart or smooth muscle revealed a band size of close to 576 bp, indicating the expression of an exon 7-containing NBCn1 variant in those tissues (data not shown). Interestingly, an additional band of smaller size, close to 160 bp, was also amplified from the mTAL tubules. This is consistent with our interpretation that renal tubules also express a splice variant which does not contain the 'A-cassette'. The expected size of the amplified message without the 'A-cassette' is 166 bp. This and all the other reported PCR products generated with the different sets of primers were sequenced and identity with NBCn1 or other transporter messages confirmed. A second set of primers was designed to investigate if the mTAL NBCn1 contains the so-called 'B-cassette' (Table 2). As shown in Fig. 2*B* we found a single band of 334 bp indicating the presence of the 'B-cassette'. In summary, our single mTAL tubule RT-PCR approach identified that the predominant variant contains the 'A-' and the 'B-cassette' (Fig. 2*D*). Furthermore, an RT-PCR approach using single isolated mTAL tubules was used to investigate if specific mRNA transcripts for other known Na^+-dependent HCO_3^- transporters were expressed in rat mTAL. As quality control, single tubules were tested in parallel for the presence of NKCC2. Two independent tubule isolation procedures showed identical results. No evidence was found for the expression of NBC4, NCBE or NDCBE in isolated mTAL segments (see Fig. 3*A*–*C*) (See different primers and gene accession nos in Table 2.) Positive control RT-PCR amplicons for NBC4 were found in choroid plexus (502 bp), for NCBE in cerebellum (498 bp) and for NDCBE in cerebellum (472 bp) (Fig. 3*A*–*C*). Clear results were obtained for the expression of the

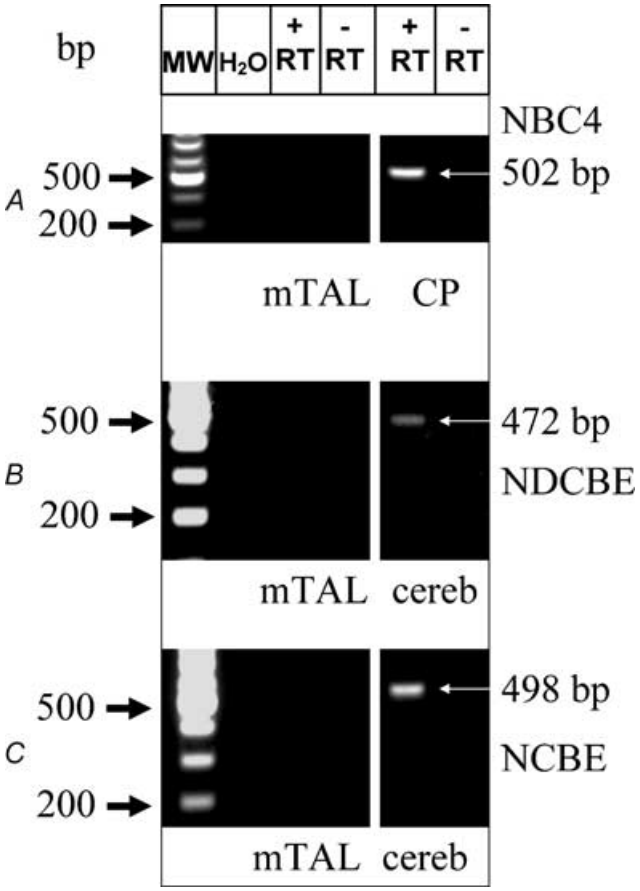

Figure 3. RT-PCR analysis of specific message for NBC4 (*A*), NDCBE (*B*) and NCBE (*C*) from 10 single mTAL tubule segments
No evidence was found for the expression of any of the three cotransporters. Positive controls for NBC4 were obtained from liver and for NDCBE and NCBE from cerebellum.

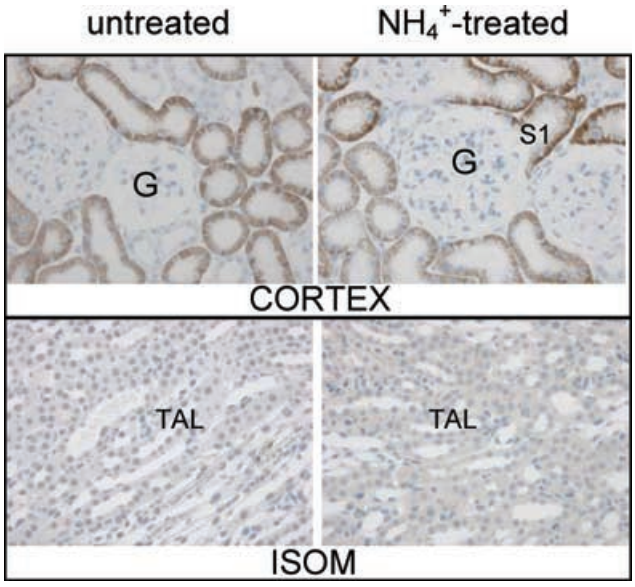

Figure 4. Immunohistochemical localization of NBCe1 in control and NH_4Cl-loaded rats
NBCe1 was localized to the basolateral plasma membrane domains of proximal tubules in the renal cortex (upper panel). No up-regulation of NBCe1 staining was observed in NH_4Cl-loaded rats. No immunolabelling of NBCe1 was seen in the same rats in the thick ascending limb in the inner stripe of the outer medulla (ISOM) or other nephron segments (lower panel). TAL, medullary thick ascending limb; G, glomerulus; S1, first segment of proximal tubule.

as yet uncharacterized BTR1 in isolated mTAL tubules, a novel member of the SLC4A solute carrier gene family (Fig. 2*C*). Despite the fact that the electrogenic Na^+-dependent HCO_3^- transporter (NBCe1) is known to be expressed only in kidney cortex (Maunsbach *et al.* 2000), we used immunohistochemistry to investigate if NBCe1 could be expressed in rat mTAL during NH_4^+-induced metabolic acidosis. The results are shown in Fig. 4. It can be clearly seen that NBCe1 is exclusively expressed in the proximal tubule and that no up-regulation occurs during metabolic acidosis. No NBCe1 labelling was observed in the inner stripe of outer medulla.

Thus our results strongly indicate that other members of the family of Na^+-coupled HCO_3^- transporters are not expressed in mTAL.

Subsequently we set out to investigate basolateral Na^+-dependent HCO_3^- import in pH_i measurements of isolated perfused mTAL segments.

Calibration of pH_i

An original trace of a single calibration experiment is shown in Fig. 5. The right panel depicts the cumulative curve of 17 experiments. Calibrations were performed at the end of the experiments. In Hepes-buffered solution (No. 1) the resting pH_i was 7.25 ± 0.04 units. In HCO_3/CO_2-containing buffer (solution No. 3) the resting pH_i was 7.1 ± 0.03 units ($n = 9$). No difference in resting pH_i was measured between normal and treated animals in the absence or presence of HCO_3^-.

Na^+-dependent recovery from acid load in HCO_3^--containing buffer

To evaluate NBCn1 function we measured basolateral Na^+-dependent import of HCO_3^- in isolated perfused mTAL segments. The following protocol as depicted in Fig. 6 was applied. It is well established in this nephron segment that removal of luminal Na^+ induces a large intracellular acidification (Sun *et al.* 1992; Watts *et al.* 1994). This pH_i acidification reflects largely the activity of luminal Na^+–H^+ (NHE3) exchange activity (Good & Watts, 1996). We therefore chose to acid-load mTAL segments by removing luminal Na^+. This also served the important purpose of excluding any effect of luminal NHE3 action when analysing Na^+-dependent recovery from acid load. As shown, removal of luminal Na^+ strongly acidified pH_i by 0.88 ± 0.11 units ($n = 10$). In NH_4^+-treated animals this pH_i decrease amounted to 0.64 ± 0.08 units ($n = 11$). Subsequently the perfusion solution on the basolateral side was changed to solution No. 4 (see Table 1) containing CO_2/HCO_3^- and amiloride (300 μM) but no Na^+. This led to a further stable acidification by 0.16 ± 0.05 pH units in control rats ($n = 10$) and 0.42 ± 0.07 pH units in NH_4^+-treated rats ($n = 11$). After 3 min, basolateral Na^+ was added in the continuous presence of basolateral amiloride (300 or 600 μM). Amiloride was used to minimize any effect of the basolateral NHE antiporter-mediated H^+ efflux. Na^+ addition in the presence of amiloride led to an instantaneous increase of pH_i. To establish the requirement for HCO_3^- in this re-alkalinization, the same experimental protocol was used

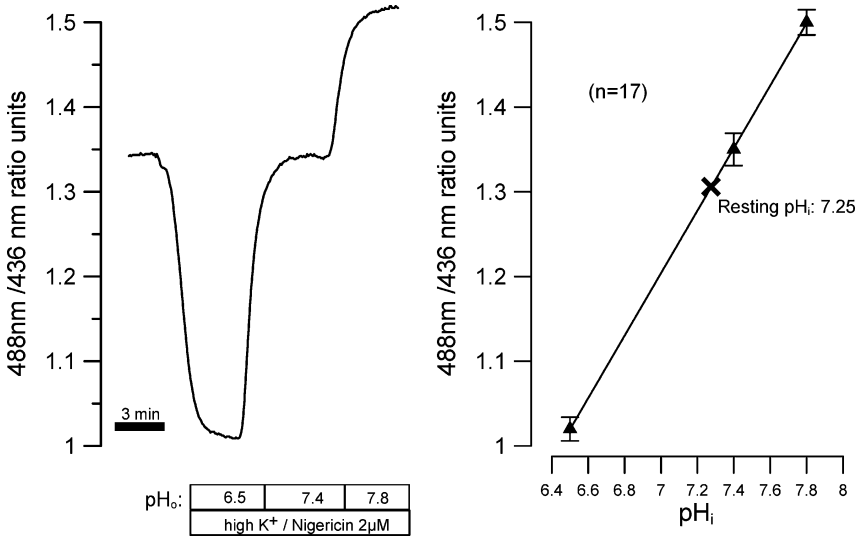

Figure 5. pH_i calibration of BCECF signal in isolated mTAL segments using the high K^+–nigericin method
Left panel, orignial BCECF fluorescence recording. Right panel, summary of 17 calibration experiments; resting pH of normal rats in Hepes buffer was 7.25

in HCO_3^--free Hepes-buffered solution (solution No. 1). An original experiment is shown in Fig. 7. The addition of Na^+ in the presence of 300 μM basolateral amiloride induced a very small re-alkalinization (0.017 ± 0.01 pH units min^{-1}, $n = 6$). In another series of experiments we tested 600 μM basolateral amiloride. Without amiloride the re-alkalinization was 0.26 ± 0.05 pH units min^{-1} and was nearly completely (94.2%) inhibited to 0.015 ± 0.14 pH units min^{-1} ($n = 6$). Thus no apparent difference was observed when using either 300 or 600 μM amiloride. To minimize any eventual effects of the luminal acid–base transport molecules (NHE3 and H^+-ATPase), these experiments were conducted in the absence of luminal Na^+ and in the presence of luminal amiloride (1 mM) and omeprazole (100 μM). Therefore the large recovery from acid load shown in Fig. 6 has an absolute requirement for HCO_3^- and thus reflects basolateral Na^+-dependent import of HCO_3^-. The recovery rate from acid load was calculated from the initial (linear) increase of pH_i. The data of the first 10–20 s were fitted to a linear function and expressed pH_i change/time. Subsequently we investigated if Na^+-dependent HCO_3^- influx is also functional at less acidic pH_i values. To impose a less dramatic acidification, two different protocols were applied and are shown in Fig. 8. After changing from a Hepes-containing buffer to a Na^+-free solution containing CO_2/HCO_3^- with 600 μM amiloride, the mTALs acidified from 7.41 ± 0.02 to 7.08 ± 0.11 pH units. Subsequent addition of basolateral Na^+ induced a rapid alkalinization (0.26 ± 0.07 pH units min^{-1}, $n = 9$). Subsequently luminal NHE3 was inhibited with amiloride (1 mM). In basolateral Na^+-free solution this led to a further acidification to 6.8 ± 0.02 units. The addition of Na^+ under these conditions again induced a rapid re-alkalinization (0.32 ± 0.13 pH units min^{-1}, $n = 4$). Therefore, Na^+-dependent HCO_3^- influx is functional at all tested pH_i values (pH_i 6.49, 6.80 and 7.08).

Figure 6. Original experiment showing the protocol used to acidify mTAL segments
See main text for detailed description. mTAL tubules were acidified by removal of luminal Na^+. Shifting basolateral Hepes buffer to CO_2/HCO_3^- buffer in the absence of basolateral Na^+ mediated a further acidification. In the presence of basolateral amiloride re-addition of basolateral Na^+ induced a rapid re-alkalinization. The dashed line indicates the initial rate of re-alkalinization.

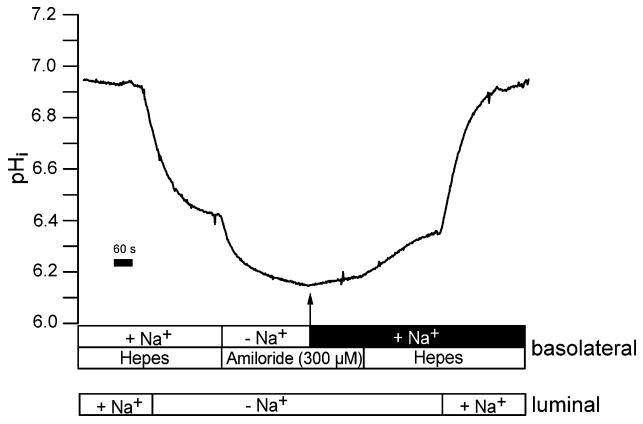

Figure 7. Absence of basolateral Na^+-dependent re-alkalinization in Hepes buffer
Original experiment of the same type as in Fig. 4 but in HCO_3^--free Hepes-containing solution. mTAL tubules were acidified by removal of luminal Na^+. Removal of basolateral Na^+ and addition of amiloride (300 μM) induced a further acidification. In the presence of basolateral amiloride, re-addition of basolateral Na^+ induced no significant re-alkalinization.

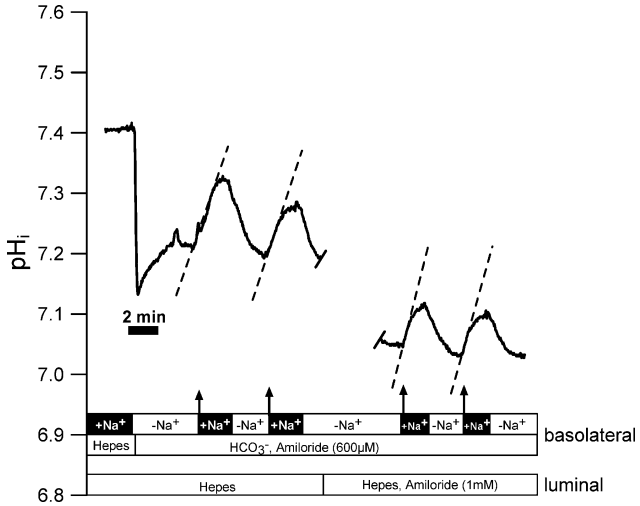

Figure 8. Na^+-dependent HCO_3^- influx is present at different pH_i values
mTAL tubules were acidified by luminal amiloride (1 mM) or in addition further acidified by basolateral amiloride (600 μM). This original trace shows a decrease of pH_i to 7.2 or 7.05, respectively. In both parts of the experiment addition of basolateral Na^+ induced a rapid and reversible re-alkalinization.

Up-regulation of Na^+-dependent HCO_3^- influx in NH_4^+-treated animals

Initially we tested whether Na^+–H^+ exchange activity was altered in control *versus* NH_4^+-treated rats. Interestingly no difference was observed. In control rats the Na^+-dependent pH_i recovery rate in Hepes-buffered solutions was 0.26 ± 0.05 *versus* 0.27 ± 0.04 pH units min^{-1} in rats treated for 7.4 ± 0.7 days with NH_4^+ ($n = 9$). These results are supported by immunolabelling results showing no change of expression of the NHE1 protein in the basolateral membrane of normal *versus* NH_4^+-treated animals (data not shown, S. Frische). The most important purpose of this study was to investigate if up-regulation of NBCn1 protein, seen in immunolabelling experiments and Western blotting (Kwon *et al.* 2002), correlates with an increase of basolateral Na^+-dependent HCO_3^- influx. Two single experiments in NH_4^+-treated and untreated animals are depicted for comparison in Fig. 9. Isolated tubules were acidified as described and shown in Fig. 6. Two significant differences between normal and NH_4^+-treated rats could be observed. (1) The initial rate of recovery (indicated by the dashed line) was significantly increased in NH_4^+-treated animals by a factor of 2.4. Recovery rates were compared at identical pH_i values. In normal rats the initial recovery rate was 0.24 ± 0.0309 pH units min^{-1} ($n = 10$) as compared to 0.64 ± 0.09 pH units min^{-1} ($n = 11$) in NH_4^+-treated animals. (2) From Fig. 9 it is also apparent that the new resting pH_i value after re-addition of Na^+ is significantly higher in NH_4^+-treated animals. In NH_4^+-treated animals the Na^+-dependent increase to a new stable pH_i value amounted to 0.38 ± 0.03 pH units ($n = 11$) as compared to 0.22 ± 0.02 pH units ($n = 10$) in untreated animals. Calculating initial J_{base} after re-addition of Na^+ (see Methods) revealed 8.4 ± 0.9 pmol min^{-1} mm^{-1} in normal and 21.8 ± 3.2 pmol min^{-1} mm^{-1} in rats treated with NH_4^+ and thus a 2.6-fold increase of HCO_3^- flux. Taken together these data indicate a strong functional up-regulation of Na^+-dependent HCO_3^- influx in NH_4^+-treated animals.

Cl^- independence of Na^+-dependent HCO_3^- influx in normal and NH_4^+-treated animals

The family of Na^+-dependent HCO_3^- transporters also encompasses Cl^--dependent transporters (NDCBE: Na^+-dependent Cl^-–HCO_3^- exchangers). We therefore repeated the experiments described in Fig. 9 in the absence of luminal and basolateral Cl^-. The same protocol was used as described for the experiments shown in Figs 6 and 9. Two original traces are shown in Fig. 10 in normal and NH_4^+-treated tubules. Cl^- was removed bilaterally 17.8 ± 3.2 min ($n = 5$) before re-addition of basolateral Na^+. Clearly it is seen that addition of Na^+ induces a rapid alkalinization and that this alkalinization is strongly up-regulated in NH_4^+-treated rats. Table 3 summarizes all results. In chloride-free conditions a 3-fold up-regulation of Na^+-dependent base influx was measured in NH_4^+-treated rats. Also in these experiments the Na^+-dependent increase to a new stable pH_i value was significantly larger in NH_4^+-treated rats (0.47 ± 0.04 pH units, $n = 5$) as compared to untreated animals (0.23 ± 0.05 pH units, $n = 5$). Table 3 shows that in both experimental series the up-regulation on Na^+-dependent HCO_3^- influx in NH_4^+-treated animals was of similar magnitude. These

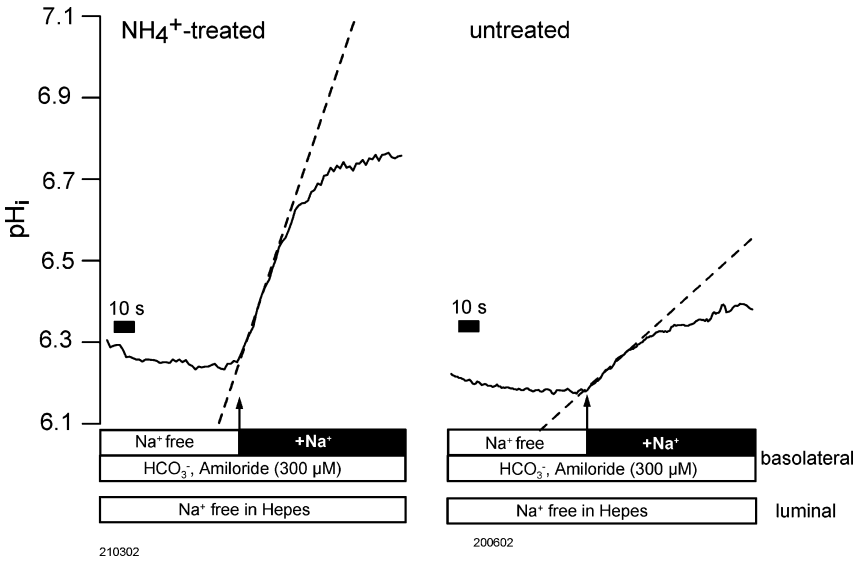

Figure 9. Comparison of Na^+-dependent re-alkalinization in normal and NH_4^+-treated animals
The protocol was as described in Fig. 4. Left panel: NH_4^+-treated animals. Right panel: untreated animals. The dashed lines indicate the initial rate of re-alkalinization. In NH_4^+-treated animals a significantly faster rate of re-alkalinization is obvious. In addition a significantly higher steady state pH_i after Na^+-dependent HCO_3^- influx is reached in NH_4^+-treated animals.

experiments clearly indicate that Na^+-dependent HCO_3^- influx occurs independently of Cl^- and thus argues for NBCn1 as the relevant transporter.

DIDS-insensitivity of basolateral Na^+-dependent HCO_3^- influx

To further characterize Na^+-dependent HCO_3^- influx we subsequently investigated its possible DIDS sensitivity. The experiments were conducted with the same protocol as those described in Fig. 6 and performed using animals treated with NH_4^+ in their drinking water for 1 week. An original experiment is shown in Fig. 11. Acid loading was again performed by removing luminal Na^+. Basolateral amiloride (300 μM) was present during the entire relevant experimental period. In CO_2/HCO_3^--containing buffer, rapid alkalinizations are visible whenever Na^+ was re-added. These Na^+-dependent rapid alkalinizations represent HCO_3^- influx. Removal of Na^+ resulted in a return to the acidic pre-control pH_i value of near 6.3. During the second addition of Na^+, DIDS (400 μM) was present (including the indicated preincubation period of 60–90 s). As shown in Fig. 11 this had no effect on Na^+-dependent HCO_3^- influx. Similar results were observed in 10 experiments (pH recovery rate before DIDS: 0.63 ± 0.11, during DIDS 0.68 ± 0.16 and after washout of DIDS 0.67 ± 0.17 pH units min^{-1}). These results indicate that basolateral Na^+-dependent HCO_3^- influx is not blocked by DIDS. DIDS-mediated inhibition of another HCO_3^- transporter located in the basolateral membrane of mTAL could, however, be shown. Medullary TAL segments also express a basolateral Cl^-–HCO_3^- antiporter, which is known to be DIDS inhibitable. Functional activity of the Cl^-–HCO_3^- antiporter can be made visible by removing basolateral Cl^-. This is known to induce rapid, reversible alkalinizations (Sun, 1998). As shown in Fig. 12, alkalinization mediated by Cl^- removal was almost completely inhibited with 400 μM DIDS ($n = 3$). These experiments strengthen the results indicating that Na^+-dependent HCO_3^- influx in rat mTAL is not blocked by DIDS.

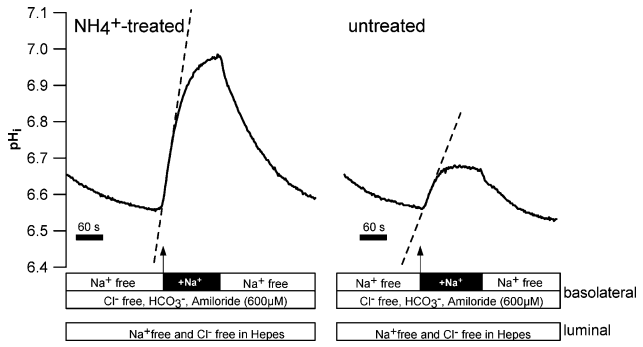

Figure 10. Comparison of Na^+-dependent re-alkalinization in normal and NH_4^+-treated animals under Cl^--free conditions
The protocol was as described in Fig. 4. Left panel: NH_4^+-treated animals. Right panel: untreated animals. The dashed lines indicate the initial rate of re-alkalinization. Also after removal of bilateral Cl^- in NH_4^+-treated animals a significantly faster rate of re-alkalinization is obvious. In addition a significantly higher steady state pH_i after Na^+-dependent HCO_3^- influx is reached in NH_4^+-treated animals.

Discussion

This study serves to increase our understanding of the recently identified electroneutral Na^+-dependent HCO_3^- transporter NBCn1 localized in rat inner stripe mTAL segments (Choi *et al.* 2000). A preceding immunolabelling study indicated the localization of NBCn1 in the basolateral membrane of mTAL segments (Vorum *et al.* 2000). Thus, the initial purpose was to identify Na^+-dependent HCO_3^- influx across the basolateral membrane of mTAL segments. This required the use of the isolated perfused kidney tubule technique to achieve sufficient control over the different luminal and basolateral acid–base transporters known in this segment. Our results identify Na^+-dependent HCO_3^- influx across the basolateral membrane of mTAL segments. We propose that this HCO_3^- influx is mediated via the NBCn1 protein. To investigate this further we studied Na^+-dependent HCO_3^- influx in normal and NH_4^+-treated animals. The addition of NH_4^+ to the drinking water is a well-established method of producing a model of chronic metabolic acidosis which has previously been shown to induce a strong up-regulation of NBCn1 protein in mTAL (Kwon *et al.* 2002). This up-regulation is confirmed in this study. We hypothesized that protein up-regulation should be reflected in the up-regulation of Na^+-dependent HCO_3^- influx. Indeed, our experiments show a strong and significant up-regulation of Na^+-dependent HCO_3^- influx in rats treated with NH_4^+. Therefore, the close correlation between up-regulation of protein and function strengthens the proposal that NBCn1 mediates Na^+-dependent HCO_3^- influx in rat mTAL. Importantly, a near to identical Na^+-dependent HCO_3^- influx in control rats was observed in the absence of Cl^-. Likewise in NH_4^+-treated rats Cl^- depletion left the strong up-regluation of Na^+-dependent HCO_3^- influx unchanged (Fig. 10 and Table 3). This strongly indicates that Na^+-dependent HCO_3^- influx in rat mTAL does not occur via the closely related Na^+-dependent Cl^-–HCO_3^- exchangers.

DIDS is a widely used inhibitor of anion and HCO_3^- transport (Cabantchik & Greger, 1992). Our study presents

Table 3. Summary of pH_i recovery rate and base flux data in normal and NH_4^+-treated rats

		Untreated	NH_4^+ treated
With chloride ($n = 11$)	pH_i recovery (pH units min^{-1})	0.24 ± 0.03	0.64 ± 0.09
	Base flux (pmol min^{-1} mm^{-1})	8.4 ± 0.9	21.8 ± 3.2
Without chloride ($n = 5$)	pH_i recovery (pH units min^{-1})	0.28 ± 0.04	0.72 ± 0.25
	Base flux (pmol min^{-1} mm^{-1})	8.7 ± 1.36	26.1 ± 9.3

evidence that the basolateral Na^+-dependent HCO_3^- influx is not blocked by DIDS. This is in close agreement with the initial cloning paper showing a very low DIDS sensitivity (Choi *et al.* 2000) Some DIDS sensitivity was, however, observed in a preceding study in which the entire kidney slice was used to measure pH_i *in situ* in mTAL segments (Kwon *et al.* 2002). Currently we do not believe that our recently shown DIDS sensitivity of Na^+-dependent recovery from acid load in whole kidney slices reflects NBCn1 function alone (Kwon *et al.* 2002). The observed DIDS insensitivity supports the interpretation that Na^+-dependent HCO_3^- influx is mediated via NBCn1.

Noteworthy is a recent study which investigated a number of basolateral acid–base transporters in isolated perfused rat mTAL segments. The authors reported Na^+-dependent HCO_3^- influx over the basolateral membrane and similarly found no effects of DIDS (Bourgeois *et al.* 2002). In addition they performed their experiments in Cl^--free medium, indicating that Na^+-dependent HCO_3^- transport occurs independently of Cl^-. Thus this study is in close agreement with our results presented here.

Is NBCn1 the only basolateral Na^+-dependent HCO_3^- importer in mTAL?

A recent publication indicates that in addition to NBCn1 yet another variant of Na^+-dependent HCO_3^- transporters, namely NBC4, could be present in rat mTAL segments as based on mRNA data (Xu *et al.* 2002). However, our RT-PCR results did not detect NBC4 message in isolated mTAL tubules. Since the rat genome has become available in 2003 we found that the primers used by Xu *et al*. to identify NBC4 in rat kidney do not recognize any relevant sequence of the rat NBC4 gene (SLC4A5). Furthermore a BLAST search revealed that the forward primer (ATGGTTGACCGATCCTTG) used by Xu *et al*. matches mouse NBC3. The reverse primer (GCTGGCTCTTAATAATGATGGC) identified 30 different sequences of diverse origin and not related to NBC sequences. We currently do not believe that rat mTAL express NBC4 and think that the above-mentioned study is faulty. In addition our RT-PCR did not show the expression of other known members of the Na^+-coupled HCO_3^- transporters NCBE (Wang *et al.* 2000; Choi *et al.* 2002) and NDCBE (Grichtchenko *et al.* 2000). Immunolabelling studies furthermore strengthened the findings that electrogenic NBCe1 is only expressed in the proximal tubules of the renal cortex and that no NH_4^+-induced expression occurs in mTAL segments. Our screen also included the functionally still uncharacterized BTR1 protein which, based on a homology analysis, was suggested to be a HCO3$^-$ transporter (Parker *et al.* 2001). As shown we could identify BTR1 message in isolated rat mTAL tubule; however, its significance is currently obscure.

As mentioned above the rat NBCn1 protein is a close homologue of the human NBC3 protein (Pushkin *et al.* 1999). Recently an NBC3 knock-out mouse was generated with the somewhat surprising phenotype of developing blindness and deafness (Bok *et al.* 2003). An apparent renal phenotype was not observed. This mouse will enable us to definitively investigate if in mTAL the NBCn1/NBC3 protein is the only Na^+-dependent HCO_3^- transporter. Nonetheless our compiled data strongly suggest that NBCn1 is probably the only protein involved in basolateral Na^+-dependent HCO_3^- uptake and is strongly up-regulated during metabolic acidosis. Our results however, do not explicitly rule out that other unforeseen candidates may contribute to this function.

Functionally basolateral Na^+–H^+ exchange (NHE1, NHE4) and Na^+-dependent HCO_3^- uptake could serve the same purpose, namely to extrude acid equivalents from the cytosol. Na^+-dependent recovery rates in the presence and absence of CO_2/HCO_3^--containing buffer allow the quantification of the contribution of the two mechanisms involved. NBC-mediated recovery in normal rats was 0.24 ± 0.03 pH units min^{-1}. In comparison NHE-mediated recovery from acid amounted to 0.26 ± 0.05 pH units min^{-1}. Interestingly, we found no up-regulation of NHE-mediated recovery from acid load in NH_4^+-treated rats. Confirmatory results were presented on the levels of the

protein and mRNA for NHE1, where no up-regulation was observed in metabolic acidosis (Laghmani *et al.* 2001). In addition, our own immunolabelling results confirm that NHE1 is not up-regulated in NH_4^+-treated animals (authors' unpublished observations).

An integrated functional role for NBCn1 in mTAL may become apparent when we consider the major functions of mTAL. As summarized in the Introduction mTAL is critically involved in NaCl, Mg^{2+}, Ca^{2+}, HCO_3^- absorption and NH_4^+ excretion. Considering the absorptive functions of mTAL, no direct involvement may intuitively be reasonable as basolateral Na^+ influx may appear counterproductive and HCO_3^- absorption requires a basolateral HCO_3^- exit step and not the contrary. However, the function of NH_4^+ excretion may indeed profit from a basolateral HCO_3^- importing mechanism. The functional concept is schematically shown in Fig. 13 and has already been mentioned in some detail in the Introduction. Metabolic acidosis is associated with increased proximal tubular formation of NH_4^+ and subsequently its handling along the tubulus (Curthoys & Gstraunthaler, 2001). It is suggested that basolateral NBCn1 serves to maintain medullary transcellular NH_4^+ shuttling by maintaining a favourable mTAL pH_i. Luminal entry of NH_4^+ via the NKCC2 transporter and the ROMK channel is well established and results in a large and continuous acid load of the mTAL cell (Good, 1994). The nature of this significant NH_4^+-induced acid load is complex, including more than the uptake of a proton via NH_4^+ (Good, 1994). An acidic pH_i of the mTAL cell will reduce the diffusable fraction of NH_3 (pK_b 9.3). On the contrary a more alkaline pH_i should significantly increase the free diffusable NH_3 and thus will allow basolateral NH_3 exit and further trapping of NH_3/NH_4^+ in the acidic collecting duct fluid. It remains to be measured more directly, however, whether functional NBCn1 is significantly required for unrestrained NH_4^+ excretion.

In summary this study strongly suggests that basolateral Na^+-dependent HCO_3^- uptake occurs via NBCn1. This Na^+-dependent HCO_3^- influx occurs independently of Cl^-. In isolated perfused mTAL segments NBCn1 was found to be DIDS insensitive. Chronic metabolic acidosis induced a strong up-regulation of NBCn1 protein and function. We suggest that NBCn1-mediated HCO_3^- uptake is an important event in transcellular transport of NH_4^+.

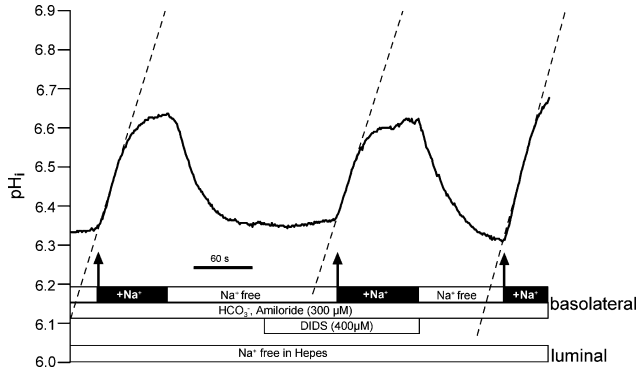

Figure 11. No effect of DIDS on Na^+-dependent re-alkalinization in an NH_4^+-treated animal

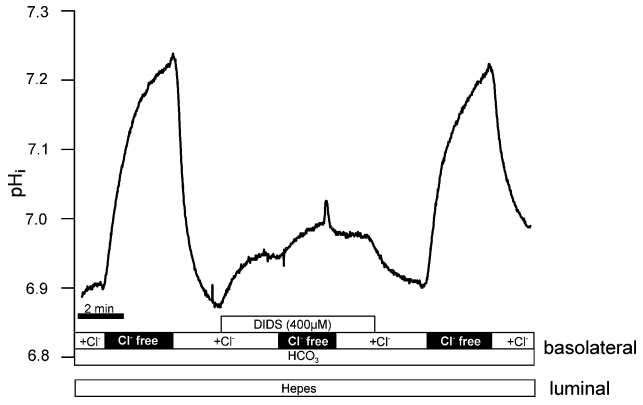

Figure 12. Effect of DIDS on alkalinization induced by removal of extracellular Cl^-

Note that DIDS had a prompt and rapid alkalinizing effect on resting pH_i. The subsequent Cl^--removal-induced alkalinization was almost completely inhibited with 400 μM DIDS. The DIDS inhibition was reversible.

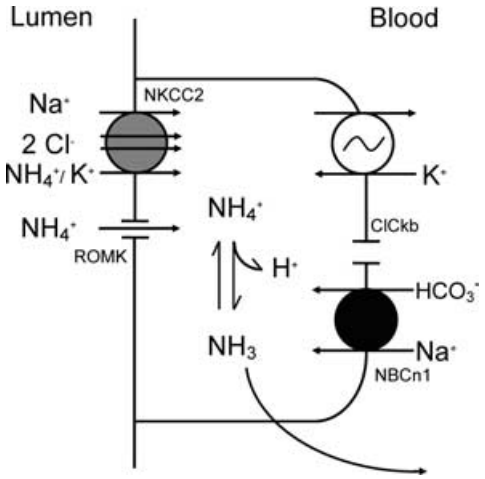

Figure 13. Schematic cell model of transcellular NH_4^+ transport in medullary thick ascending limb

NBCn1 is suggested to be important for buffering the acid load imposed on the cell by uptake of luminal NH_4^+ via the NKCC2 transporter and ROMK channels.

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